

**Transcriptional regulation of the glucoamylase-encoding
gene under endoplasmic reticulum stress in *Aspergillus
niger***

Hashem M. Al-Sheikh (M.Sc.)

A thesis submitted to the University of Nottingham for the degree of
Doctor of Philosophy

(February 2005)

Transcriptional regulation of the glucoamylase-encoding gene under endoplasmic reticulum stress in *Aspergillus niger*

Abstract

The ability of many filamentous fungi, such as *Aspergillus niger*, to secrete a high level of homologous proteins has led to their consideration as hosts for the production of heterologous proteins. However, the levels of some secreted heterologous proteins have often been low. Although many strategies have been developed to improve the level of secreted heterologous proteins, further studies into the remaining bottlenecks are required. One common strategy used to improve secreted protein production from filamentous fungi is to express the target protein under the control of a highly-induced native promoter. One major bottleneck in the secretion of heterologous proteins is caused by the folding of proteins within the lumen of the endoplasmic reticulum (ER). Recent studies have shown that expressing some heterologous proteins could subject *A. niger* to ER-stress. In this study, *A. niger* was subjected to different environmental conditions and ER stress responses were examined under each of these environmental conditions to further investigate the regulation of the gene encoding glucoamylase (*glaA*). Treating *A. niger* with dithiothreitol (DTT), a reducing agent that causes the formation of unfolded proteins, caused the down-regulation of transcription of the *glaA* but not the gene encoding the non-secreted protein γ -actin. The DTT-treated fungal cells also showed evidence of induction of the UPR because expression of *bipA* was up-regulated and splicing of *hacA*, the gene encoding the transcription factor responsible for induction of the Unfolded Protein Response (UPR), occurs allowing the production of an active HacA protein. This is the first study to show clearly by nuclear run-on studies that the transcriptional down-regulation effect occurs at the level of transcription, rather than mRNA stability, and is found to be mediated through the promoter of the *glaA* gene (P_{glaA}) in a region more than 1.192 kb upstream of the translational start.

As a preliminary attempt to investigate if the transcriptional down-regulation effect was mediated through HacA (i.e. part of the UPR), the ER stress was induced through antisense technology to lower the level of PDI in the ER of *A. niger*. Although the transcription of *glaA* was attenuated in that strain of *A. niger*, UPR was not evident, suggesting that the transcriptional down-regulation mechanism is controlled differently from the UPR.

Furthermore, activation of the ER-Associated Degradation (ERAD) mechanism in DTT-treated *A. niger* cultures was demonstrated by detecting transcriptional up-regulation of the putative gene encoding the RpnG, a homologue of the yeast Rpn7p subunit of the 26S proteasome.

Acknowledgments

I would like to thank my supervisor, Professor David Archer, for his invaluable advice and guidance throughout my PhD. I am also grateful to King Faisal University for funding my research.

Many thanks to the members of the *Aspergillus* group and my friends at Nottingham University for their help and cooperation during my study.

And to my parents, Sultanah and Mohammed, for their continuing love and support.

Finally, I would like to say that I cannot thank enough Omaimah for her love and encouragement, and Sultan and Sultanah for their presence in my life.

Dedicated to my parents, my wife Omainah, and my children, Sultan and Sultanah

Selected publications

Al-Sheikh, H., A. J. Watson, G. A. Lacey, P. J. Punt, D. A. MacKenzie, D. J. Jeenes, T. Pakula, M. Penttilä, M. J. C. Alcocer and D. B. Archer. (2004) Endoplasmic reticulum stress leads to the selective transcriptional downregulation of the glucoamylase gene in *Aspergillus niger*. *Mol. Microbiol.* **53**, 1731-1742.

D. A. MacKenzie, **H. Al-Sheikh**, A. J. Watson, D. J. Jeenes, P. Wongwathanarat, N. Dunn-Coleman and D. B. Archer. Dithiothreitol stress-induced genes in *Aspergillus niger*. identification of two 26S proteasomal subunit genes, *rpnG* and *rpnI*. Submitted to *Mol. Gen. Genom.*

Al-Sheikh, H., D. J. Jeenes, A. J. Watson, M. J. C. Alcocer, P. J. Punt, and D. B. Archer. (April, 2003) Protein secretion stress in *Aspergillus niger*. *Fungal Genetics Newsletter*, Vol. 50, Suppl., Proc. XXII Fungal Genetics, Asilomar, USA. p. 82.

Al-Sheikh, H., and D. B. Archer. (February, 2004) Endoplasmic reticulum stress leads to the selective transcriptional down-regulation of the glucoamylase gene in *Aspergillus niger*. EUROFUNG, The Genomic Era. WICC, Wageningen, The Netherlands, p. 28.

Al-Sheikh, H., A. J. Watson, G. A. Lacey, P. J. Punt, D. A. MacKenzie, D. J. Jeenes, T. Pakula, M. Penttilä, M. J. C. Alcocer and D. B. Archer. (April, 2004) Endoplasmic reticulum stress leads to the selective transcriptional downregulation of the glucoamylase gene in *Aspergillus niger*. Proceedings of the the 7th European Conference on Fungal Genetics, Copenhagen, Denmark, p. 196.

Al-Sheikh, H., A. J. Watson, G. A. Lacey, P. J. Punt, D. A. MacKenzie, D. J. Jeenes, T. Pakula, M. Penttilä, M. J. C. Alcocer and D. B. Archer. (September, 2004) Endoplasmic reticulum stress leads to the selective transcriptional down-regulation of the glucoamylase gene in *Aspergillus niger*. BMS meeting, Fungi in the Environment. University of Nottingham, UK, p. 79.

Archer, D. B., M. J. C. Alcocer, **H. Al-Sheikh**, S. Barnes, and A. J. Watson, (July, 2003). The secretion of heterologous proteins by fungi. Proceedings of the MSA/BMS Symposium, Asilomar, USA. P. 32

Abbreviations

aa	amino acid(s)
AAB	Am Alpha Binding protein
AARE	amino acid response element
ACM	<i>Aspergillus</i> complete medium
ACMS/N/P	ACM with starch as carbon source/ammonium chloride/phosphate buffer
ACMG/N/P	ACM with glucose as carbon source/ammonium chloride/phosphate buffer
ACMX/N/P	ACM with xylose as carbon source/ammonium chloride/phosphate buffer
<i>aglA</i>	gene encoding guar α -galactosidase
<i>Am</i>	gene encoding the anabolic NADP-specific glutamate dehydrogenase
AMMN	<i>Aspergillus</i> minimal medium with ammonium chloride
<i>A. niger</i>	<i>Aspergillus niger</i>
<i>A. thaliana</i>	<i>Arabidopsis thaliana</i>
ATF6	Activating Transcription Factor 6
ATP	adenosine 5'-triphosphate
BFA	brefeldin A
Bip	immunoglobulin heavy chain binding protein
bp	base pair

BSA	bovine serum albumin
<i>cbh1</i>	gene encoding cellobiohydrolase I
Chs	chitin synthesis
COP	coatomer
CPA	core promoter activation
CP	core particle
CREB	cAMP responsive element binding protein
dctp	2'-deoxycytidine 5'-triphosphate
Dgt	delayed GPI-anchored protein transport
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
eIF2 α	α subunit of eukaryotic initiation factor 2
EMSA	electrophoretic mobility shift assay
ER	endoplasmic reticulum
ERSE	endoplasmic reticulum stress response element
ER-QC	endoplasmic reticulum-quality control
GAM	glucoamylase
Gap1p	general amino acid permease in <i>Saccharomyces cerevisiae</i>
GFP	green fluorescent protein
<i>glaA</i>	gene encoding glucoamylase
<i>gpdA</i>	gene encoding glyceraldehyde-3-phosphate dehydrogenase
GlcNAc-T1	N-acetylglucosaminyltransferase

GPI	glycosyl phosphatidylinositol-linked protein
GRP	glucose regulated protein
HEWL	hen egg-white lysozyme
HSP	heat shock protein
<i>KAR2</i>	gene encoding Bip in <i>S. cerevisiae</i>
kb	kilobase
kD	kilodaltons
Lag	longevity-assurance gene
LB	Luria-Bertani
LST	lethal with sec-thirteen
MHC	major histocompatibility complex
MOPS	3-(N-morpholino) propanesulphonic acid
mtDNA	mitochondrial DNA
rDNA	ribosomal DNA
MUG	4-Methylumbellifery- β -D-glucuronide
NSF	<i>N</i> -ethylmaleimide-sensitive fusion protein
nt	nucleotide
ORF	open reading frame
PCR	polymerase chain reaction
PDI	protein disulphide isomerase
Pep	aspergillopepsin
<i>pepA</i>	gene encoding aspergillopepsin A in <i>A. niger</i>
PIPES	Piperazine-1,4-bis(2-ethanesulfonic Acid)

PLA ₂	porcine pancreatic phospholipase A ₂
PMSF	phenylmethanesulphonyl fluoride
PPIase	peptidyl prolyl isomerase
PRAD1	parathyroid adenomatosis 1
<i>pyrG</i>	gene encoding orotidine 5'decarboxylase in <i>A. niger</i>
RAP	receptor-associated protein
RACE	rapid amplification of cDNA ends
RP	regulatory particle
rpm	revolutions per minute
RT	room temperature
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SEM	standard error of the mean
SDS	sodium dodecyl sulphate
Ser	serine
SNAP	soluble NSF associating-proteins
Thr	threonine
uORF	upstream open reading frame
XBP	<u>X</u> -box <u>b</u> inding <u>p</u> rotein
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
UPR	unfolded protein response
UPRE	unfolded protein response element
wt	wild type
w/v	weight/volume

Table of contents

1 General Introduction	Page
1.1 Filamentous fungi as hosts for the production of heterologous proteins	1
1.1.1 Improvement strategies to overcome limitations in using filamentous fungi as hosts for heterologous protein production	7
1.2 Secretion pathway	14
1.2.1 Endoplasmic reticulum (ER) targeting	15
1.2.2 Protein modifications in the ER	19
1.3 The Unfolded Protein Response (UPR)	30
1.3.1 Activation of the UPR mechanism in <i>S. cerevisiae</i>	31
1.3.2 Activation of the Unfolded Protein Response (UPR) mechanism in filamentous fungi	40
1.4 Aim of the research and outline of the thesis	43
 2 General Materials and Methods	
2.1 Chemicals and reagents	45
2.2 Strains	45
2.3 Strain maintenance, media, and growth conditions	45
2.4 Harvest of mycelia	46
2.5 Isolation and manipulation of DNA	47
2.6 Polymerase Chain Reaction (PCR)	48
2.7 RNA extraction	48
2.8 Northern blots and quantification of transcript levels	48
2.9 Probing and hybridisation of the blots	49
2.10 β -Glucuronidase activity assay	50

2.11	Assay of total protein concentration	51
2.12	Nomenclature of strains	51
3	Activation of ER stress responses	
3.1	Introduction	52
3.2	Materials and Methods	
3.2.1	Primers used for preparation of DNA probes for northern analysis of transcripts levels	54
3.2.2	Isolating mRNA and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)	54
3.2.3	Medium-exchange for improving tunicamycin treatment	55
3.2.4	Isolation of poly ⁺ A-RNA (including mRNA) for northern blots	56
3.2.5	Media and growth conditions for studying the impact of low and high nitrogen concentration in the growth media on <i>hacA</i> unconventional intron splicing	56
3.3	Results	
3.3.1	Decline in the transcript levels of the indigenous glucoamylase-encoding gene in DTT-treated cultures of <i>A. niger</i> AB4.1	57
3.3.2	Activation of the UPR mechanism in DTT-treated <i>A. niger</i> AB4.1 cultures	59
3.3.3	Treatment of <i>A. niger</i> cultures with tunicamycin results in poor activation of the UPR compared to DTT	62

3.3.4	No obvious splicing of the unconventional <i>hacA</i> mRNA intron in <i>A. niger</i> strains AS1.1, ASG67 both with reduced level of PdiA, and <i>A. niger</i> B1 producing a heterologous protein	67
3.3.5	No obvious splicing of the unconventional <i>hacA</i> mRNA intron in <i>A. niger</i> AB4.1 cultures grown in medium with low and high concentrations of nitrogen	69
3.3.6	Activation of the ER-Associated Degradation (ERAD) mechanism in DTT-treated <i>A. niger</i> AB4.1 cultures	71
3.4	Discussion	76

4 Role of the glucoamylase promoter (P_{glaA}) under ER stress

4.1	Introduction	81
4.2	Materials and Methods	
4.2.1	<i>A. niger</i> reporter strains with different P_{glaA} truncation	87
4.2.2	RT-PCR primers	87
4.2.3	Preparation of DNA probes for Ribonuclease Protection Assay (RPA)	90
4.2.4	Radiolabel incorporation test for synthesized RNA probes	90
4.2.5	Ribonuclease Protection Assay (RPA)	94
4.2.6	Preparation of and running the denaturing polyacrylamide gels	94
4.2.7	Testing for DTT interference with the fluorimetric β -glucuronidase Assay	94

4.3	Results	
4.3.1	DNA sequence of the truncated glucoamylase promoter (P_{glaA}) used in the <i>A. niger</i> reporter strains	96
4.3.2	The <i>uidA</i> expression level in <i>A. niger</i> reporter strains GUS64, GUS64 <i>SalI</i> , GUS64 <i>BamHI</i> , and GUS64 <i>MluI</i> grown in different carbon sources	96
4.3.3	Positive and negative controls used in RPA showed the expected patterns	102
4.3.4	Effects of DTT treatments on the transcript levels of the <i>uidA</i> reporter gene and the gene encoding the molecular chaperone <i>bipA</i> in <i>A. niger</i> reporter strains GUS64, GUS64 <i>SalI</i> , GUS64 <i>BamHI</i> , and GUS64 <i>MluI</i>	105
4.3.5	DTT interferes with the fluorimetric β -glucuronidase assay	114
4.3.6	Stability of the <i>uidA</i> transcripts in <i>A. niger</i> GUS64 and GUS64 <i>SalI</i> strains is similar under different growth conditions	119
4.3.6.1	Stability of <i>uidA</i> mRNA transcripts and β -glucuronidase after switching to a non-inducing carbon source of the <i>glaA</i> promoter (P_{glaA})	119
4.3.6.2	Size of <i>uidA</i> cDNA under DTT treatment and medium- exchange is similar in <i>A. niger</i> GUS64 and GUS64 <i>SalI</i> strains	125
4.4	Discussion	130

5	Transcriptional down-regulation of <i>glaA</i> under secretion stress at the nuclear level	
5.1	Introduction	136
5.2	Materials and Methods	
5.2.1	Media-exchange and growth conditions	138
5.2.2	Nuclear run-on Assay	139
5.2.2.1	Isolation of the nuclei	139
5.2.2.2	Nuclear run-on transcription assay	139
5.2.2.3	Hybridisation of DNA dot blot	140
5.2.2.4	Washing and exposing DNA dot blot	140
5.3	Results	
5.3.1	Transcription of <i>uidA</i> following medium-exchange	141
5.3.2	Transcriptional regulation of the <i>uidA</i> -encoding gene in different <i>A. niger</i> reporter strains under ER-stress conditions	147
5.3.3	The mechanism of transcriptional down-regulation under secretion stress is a feedback mechanism that functions at the transcriptional level	150
5.3.4	Effect of DTT treatment on the viability of <i>A. niger</i> cultures	154
5.3.5	DTT leads to general transcriptional repression	157
5.4	Discussion	159

6	General discussion	163
	References	179

1 General Introduction

1.1 Filamentous fungi as hosts for the production of heterologous proteins

Filamentous fungi are widespread in nature and include many species that are saprophytic. Many of these filamentous fungal species secrete a wide variety of enzymes capable of degrading a large diversity of biopolymers (Oxenboll, 1994). One example of an extensively-used filamentous fungus for the production of fungal enzymes is *Aspergillus niger* (MacKenzie *et al.*, 2004). *A. niger* has been classified as a member of the deuteromycetes (Fungi Imperfecti), as no sexual stage has been identified in the life cycle of this fungus. *A. niger* and its close relatives form black conidial heads and because of this morphological property, they are sometimes referred to as the black Aspergilli (Kusters-van someren *et al.*, 1990). Furthermore, classification studies of *Aspergillus* using molecular biological techniques including G + C molar percentage, DNA:DNA complementarity, ribosomal RNA sequence comparison, and restriction fragment length polymorphism (AFLP), have shown that all black *Aspergillus* isolates examined are related (Klich *et al.*, 1993; Kumeda and Asao, 2001). For example, black *Aspergillus* isolates were examined by restriction fragment length polymorphisms (RFLPs) using their mitochondrial DNAs (mtDNAs) and ribosomal DNA (rDNA). Most isolates were classifiable as *A. niger* or *Aspergillus tubingensis* according to their rDNA and mtDNA patterns (Varga *et al.*, 1994).

The ability to genetically modify filamentous fungi and the ability of fungi such as *A. niger*, *Aspergillus oryzae* and *Trichoderma reesei* to secrete a high level of different enzymes has stimulated the industrial application of these species (van Gorcom *et al.*, 1994). In fact there are several reasons for using filamentous fungi as hosts for the production of recombinant proteins. Table 1.1 summarizes some of the reasons for using filamentous fungi as hosts for the production of recombinant proteins. Although many of the food safety studies used as sources for the information in Table 1.1 were performed specifically on *A. niger* (Schuster

et al., 2002), other *Aspergillus* species, such as *A. oryzae*, have been used for a long time and a number of their products have been generally recognized as safe (GRAS) by the US Food and Drug Administration (FDA) (Oxenboll, 1994).

Table 1.1 Reasons for using *A. niger* as a host for the production of recombinant proteins

Reason	Reference
The ability to secrete high amounts of a wide range of different enzymes	(Jeenes <i>et al.</i> , 1991)
The ease of both handling the cultures and recovering the products, and the ability to grow rapidly on cheap substrates	(Oxenboll, 1994)
Long experience of industrial use which results in a good knowledge of the characteristics of the microorganism and understanding of the metabolic reactions	(Anke, 1997)
Information about <i>Aspergillus</i> genome sequences and methods for exploiting that information are becoming available	(Groot <i>et al.</i> , 2002) (Archer and Dyer, 2004)
Many enzymes produced from <i>A. niger</i> have been in the market for a long time and many have GRAS status from the US Food and Drug Administration (FDA)	(Schuster <i>et al.</i> , 2002) (Oxenboll, 1994)
Many genetically-modified host strains of <i>A. niger</i> which can overexpress various recombinant proteins are available and have been approved as GMO self-clones ^a	(van Dijck <i>et al.</i> , 2003)
No harmful components could be detected from recombinant proteins produced by tested <i>A. niger</i> strains where target genes were integrated either randomly or at specific loci in the genome	(van Dijck <i>et al.</i> , 2003)

^a Self-cloning as defined in the European Directive on the Contained Use of Genetically Modified Micro-organisms 98/81/EC (revision of 90/219/EC).

There is a wide range of applications where filamentous fungi are used. For instance, filamentous fungi have been used in the production of antibiotics, organic acids, vitamins, polysaccharides, extracellular enzymes and food (Anke, 1997; Archer, 2000). Some of the recent examples for heterologous proteins,

either of fungal or non-fungal origin, are shown in Table 1.2. However, in most cases the secreted yields of heterologous proteins are disappointingly low compared to the yields of many homologous proteins.

Production of high quantities of heterologous proteins generates the need to genetically improve the fungal host strain under protein secretion stress conditions where the secretion capacity of the host is limited or the secretion route overloaded. The filamentous fungus *T. reesei* strain, producing the heterologous protein human tissue plasminogen activator (t-PA), a serine protease, secreted much less total protein and cellulase activity into the culture medium compared to its parental strain (Pakula *et al.*, 2001). Furthermore, expression of t-PA in *A. niger* was shown to activate a feedback mechanism known as the Unfolded Protein Response (see below), indicating that the *A. niger* strain was subjected to ER-stress (Mulder *et al.*, 2004; Wiebe *et al.*, 2001). ER-stress can also result from treating the cell with a chemical agent that can hinder the synthesis, folding or transport of the protein (e.g. dithiothreitol (DTT), a reducing agent (Alberini *et al.*, 1990; Braakman *et al.*, 1992)), tunicamycin, an inhibitor of N-glycosylation, or a chemical that perturbs the calcium balance in the endoplasmic reticulum (ER) such as the ionophore A23187 (Lodish and Kong, 1990; Wileman *et al.*, 1991) and which leads to the accumulation of unfolded proteins in the ER. This accumulation of unfolded proteins in the ER activates the UPR (described in section 1.3.2), which leads to the induction of a number of genes that encode proteins involved in protein folding, glycosylation, transport, and degradation of misfolded proteins (Mori, 2000a; Travers *et al.*, 2000).

Table 1.2 Recent examples of heterologous (fungal and non-fungal origins) proteins from filamentous fungi. The Table was adapted from MacKenzie et al. (2004)

a. Fungal proteins

Expression host	Source	Enzyme	Reference
<i>A. niger</i>	<i>Caldariomyces fumago</i>	Peroxidase	(Conesa et al., 2001b)
<i>A. niger</i>	<i>Phanaerochaete chrysosporium</i>	Peroxidase	(Conesa et al., 2000)
<i>A. niger</i>	<i>Williopsis markii</i>	Mycocin	(Lowes et al., 2000)
<i>A. niger</i>	<i>Pleurotus eryngii</i>	Peroxidase	(Ruiz-Duenas et al., 2001)
<i>A. oryzae</i>	<i>Aspergillus aculeatus</i>	Arabinase	(Skjot et al., 2001)
<i>A. oryzae</i>	<i>Aspergillus fumigatus</i>	Naphthopyrone synhase	(Watanabe et al., 2000)
<i>T. reesei</i>	Bacteria	Xylanase	(Paloheimo et al., 2003)

b. Non-fungal proteins

Expression host	Source	Protein	Reference
<i>A. niger</i>	Human	Tissue Plasminogen activator	(Wiebe et al., 2001)
<i>A. niger</i>	Human	Tumour necrosis factor	(Krasevec et al., 2000a)
<i>A. niger</i>	Human	Lymphotoxin	(Krasevec et al., 2000b)
<i>A. niger</i>	Human	Lysozyme	(Canet et al., 2002)

The UPR mechanism is activated in the cell, most likely as a defence mechanism, to alleviate the harmful effects of the limited capacity of the secretory pathway. Several strategies based on gene manipulation, reported to improve secreted protein production from filamentous fungi have recently been reviewed (MacKenzie *et al.*, 2004) (Table 1.3). In this section, approaches to overcome limitations in using filamentous fungi as hosts for the production of recombinant proteins, both homologous and heterologous, will be discussed.

Table 1.3 Gene manipulation strategies to overcome limitations in using filamentous fungi as hosts for heterologous protein production. The Table was adapted from MacKenzie *et al.* (2004)

Strategy	Expected benefits	Possible limitations
Gene dosage	Maximise transcription	Titration of transcription factor. Concomitant enhancement of transcription factors has not been tried in filamentous fungi. Gene integration is potentially mutagenic.
Gene fusion	Encourage secretion and, possibly, improve mRNA stability	Requires cleavage (might be achieved by introducing a proteolytic cleavage site) to separate target protein from carrier protein. This may not be wholly accurate, depending on the fusion.
Protease-deficient strain	Reduce proteolytic degradation of target protein	Other proteases may be present
Strong native promoter	Maximise transcription	Promoter may not be as effective with non-native gene

1.1.1 Improvement strategies to overcome limitations in using filamentous fungi as hosts for heterologous protein production

One important characteristic of filamentous fungi is their ability to secrete high levels of homologous proteins. For example, *A. niger* and *T. reesei* have been reported to secrete 20g glucoamylase/litre and 40g cellulase/litre respectively, under optimal growth conditions (Durand *et al.*, 1988; Finkelstein *et al.*, 1989). However, in most cases the secreted yields of heterologous proteins are below gram/litre (Davies, 1994; Gouka *et al.*, 1996a; van Gorcom *et al.*, 1994). Several strategies have been developed to improve the secreted yields of heterologous proteins from fungi (Archer *et al.*, 1994a; Archer and Peberdy, 1997; MacKenzie *et al.*, 2004). These strategies mainly include increasing gene dosage (Verdoes *et al.*, 1994), gene fusion (Nyyssönen and Keränen, 1995; Tsuchiya *et al.*, 1994; Ward *et al.*, 1990), and the use of protease-deficient strains (Archer *et al.*, 1994a; van den Hombergh *et al.*, 1997). In these strategies the target gene is commonly expressed under the control of a strong native promoter.

A. Gene dosage

One of the strategies to improve the production of recombinant proteins is to increase the transcript levels of the target gene by increasing the copy number of that target gene. It has been shown that integration of multiple copies of the target gene can increase the protein level (Verdoes *et al.*, 1993). Using a reporter strain containing a fusion of the glucoamylase promoter (P_{glaA}) of *A. niger* to the β -glucuronidase-encoding gene (*uidA*) of *Escherichia coli*, it was shown that increasing the number of expression cassettes up to about 20 copies results in an increase of the *uidA* expression (Verdoes *et al.*, 1994). A similar increase was seen for glucoamylase expression levels in the *A. niger* strain containing multiple copies of the *glaA* gene (Verdoes *et al.*, 1993). However, in both studies introduction of higher copy numbers of the target gene resulted in a decrease of the expression of the target genes under the control of P_{glaA} . It was concluded that

introduction of multiple copies of P_{glaA} (more than 20 copies) causes titration of *trans*-acting regulatory protein(s) (Verdoes *et al.*, 1994). Furthermore, a limitation of transcriptional regulatory factors were observed in another filamentous fungus, *A. nidulans*, when overexpressing some genes such as *qutE*, a gene encoding catabolic 3- dehydroquinase (Beri *et al.*, 1990), and *amdS*, a gene encoding acetamidase (which hydrolyses acetamide to acetate and ammonium permitting utilisation of acetamide as a sole carbon and/or nitrogen source, (Andrianopoulos and Hynes, 1988)). Thus, it may sound logical to overcome the limitation in the transcriptional regulatory factors by overexpressing genes encoding these transcriptional factors by increasing their gene copy number (Beri *et al.*, 1990). Furthermore, a transcriptional activator gene, *amyR*, involved in the amylolytic gene expression in *A. oryzae*, was cloned by anti-titration of the limiting transcription factor (Gomi *et al.*, 2000). This was facilitated by preparing a genomic library by shot-gun cloning of the activator gene and screening for a clone that enabled a reversing of the titration effects. So this observation may suggest the possibility of overexpression of the transcription factors as a strategy to overcome the titration effect.

B. Gene fusion

One of the strategies that improves the yields of heterologous proteins is the fusion of a well-secreted carrier protein to the N-terminal end of a heterologous protein. The common carrier proteins used in filamentous fungi are *A. niger* glucoamylase (Broekhuijsen *et al.*, 1993; Contreras *et al.*, 1991; Ward *et al.*, 1990; Ward *et al.*, 1995) or *T. reesei* cellobiohydrolase I (Nyyssönen and Keränen, 1995). The N-terminus of the carrier protein is believed to improve the secretion efficiency of the heterologous protein by facilitating translocation and processing in the ER. Furthermore, the N-terminal fusion not only aids secretion, but also may increase the level of the heterologous mRNA either by stabilising it or by enhancing transcription (Nyyssönen and Keränen, 1995). The fusion protein, in most cases, is cleaved, resulting in secretion of the separate proteins. The fusion is engineered with a kexin endoproteolytic cleavage site (Lys-Arg preferred) at the

junction of the carrier and target proteins. The carrier protein is one which is naturally well-secreted by the fungus, such as glucoamylase or cellobiohydrolase. The cleavage occurs within the late secretory pathway and is rarely aberrant (Archer, 2000). Recently, a kexin-encoding gene *kexB* has been cloned from *A. niger*, and it was shown that KexB is an endoproteolytic proprotein processing enzyme responsible for the processing of dibasic cleavage sites in target proteins that are transported through the secretion pathway (Jalving *et al.*, 2000; Punt *et al.*, 2003). Overexpression of *kexB* only results in a small increase in the activity of KexB, suggesting a regulatory mechanism operative at the post-transcriptional level. This suggestion is supported by the observation that, when the Kex2 protease was overexpressed in *S. cerevisiae*, it was transported to the vacuole at an increased rate and degraded (Wilcox *et al.*, 1992). In *A. niger* a *kexB* disruptant could not process the fusion protein, and grew very poorly and had an unusual morphology on agar plates. In shake flask cultures, the *kexB* disruptant had less severe morphology changes and no reduction in the biomass could be detected (Jalving *et al.*, 2000). On the other hand, no unusual phenotype change was detected with strains overexpressing *kexB*. Some examples in filamentous fungi where the gene fusion strategy results in higher secreted levels of target heterologous protein include bovine prochymosin (Ward *et al.*, 1990), porcine pancreatic phospholipase A₂ (Roberts *et al.*, 1992), human interleukin-6 (Contreras *et al.*, 1991), hen egg-white lysozyme (Archer *et al.*, 1990a), and human lactoferrin (Ward *et al.*, 1995).

C. Protease-deficient strains

One of the well-recognised problems that limits efficient production of heterologous proteins is the action of proteases. The yields of two heterologous proteins, hen egg-white lysozyme (HEWL) and porcine pancreatic phospholipase A₂ (PLA₂) were found to be limited by protease when expressed in *A. niger* (Archer *et al.*, 1992). HEWL protein was found to be readily degraded by mycelium; however, it was stable in the culture filtrates. On the other hand, PLA₂ was degraded both in the culture filtrate and mycelia. When an *A. niger* mutant strain

deficient in the extracellular protease aspergillopepsin A (PepA) was used, PLA₂ was still degraded, albeit more slowly, but PLA₂ was much more stable in culture filtrates of an *A. niger* mutant strain lacking both aspergillopepsin A and B (Mattern *et al.*, 1992). The use of the protease-deficient strains has been shown to be an effective strategy for improving the yield of heterologous proteins. Berka *et al.* (1990) demonstrated that disruption of the major extracellular acid protease gene *pepA* in *A. niger* reduced extracellular proteolytic activity to approximately 20% compared to the wild-type strain (Berka *et al.*, 1990). Disruption of the *pepA* gene in another strain of *A. niger* results in similar observations (Mattern *et al.*, 1992). Analysis of the spectrum of proteases in *A. niger* revealed that acid proteases predominate. The proteases identified in *A. niger* so far include PepA (extracellular endoprotease), PepE (intracellular endoprotease), PepB (extracellular endoprotease), PepC (intracellular endoprotease), PepD (extracellular endoprotease), PepF (extracellular endoprotease), PepG (extracellular endoprotease), CpY (intracellular endoprotease), and Pept (extracellular endoprotease) (van den Hombergh *et al.*, 1997). Four of those proteases, PepA, PepB, PepF, and PepG, have acid pH optima for activity. This may explain why in *A. niger*, which is known for its strong acidification of culture media, acidic protease activities cause the major problem during heterologous protein expression. In addition to the protease-deficient strains strategy, another strategy is to down-regulate the expression of protease-encoding genes by altering the growth medium composition. It was found that depletion of the low molecular weight sources of nitrogen, carbon, phosphorus and sulphur resulted in elevated protease activities in *A. oryzae* and *A. nidulans* (van den Hombergh *et al.*, 1997). Furthermore, northern expression studies in *A. niger* showed that extracellular proteases-encoding genes (*pepA*, *pepB*, *pepD*, and *pepF*) are only expressed when the preferred carbon and nitrogen sources are not available to the cell (van den Hombergh *et al.*, 1997). Also, the *pepF* gene is repressed by glucose, ammonia, and alkaline pH (van den Hombergh *et al.*, 1994). These observations have led to the analysis of the effect of disrupting genes encoding wide-domain regulatory proteins. In *A. niger*, disruption of three regulatory genes,

creA (encoding a negative regulatory protein CreA that mediates the carbon catabolite repression (Drysdale *et al.*, 1993)), *areA* (encoding a regulatory protein involved in nitrogen metabolite repression) and *pacC* (encoding a protein mediating pH-regulation of transcription (MacCabe *et al.*, 1996) have been studied. Mutant strains containing disruptions of these genes showed strongly reduced transcription levels of all extracellular proteases and a reduction in their activity (van den Hombergh *et al.*, 1997). Moreover, consensus recognition motifs for CreA and AreA were found in the promoter regions of *pepA*, *pepB* and *pepF* genes in *A. niger* but they were not functionally assessed (Archer and Peberdy, 1997). The use of protease-deficient strains is likely to be an effective strategy for improving heterologous yields. However, yield reduction of target heterologous proteins can occur *in vivo* by intracellular degradation (Archer *et al.*, 1992; Gouka *et al.*, 1997a), which would limit the use of protease inhibitors to eliminate the proteolysis problem. Furthermore, the addition of protease inhibitors to the growth media is unlikely to be a cost-effective measure commercially (Archer and Peberdy, 1997), thus emphasizing the need for strain modification to improve the production of heterologous proteins.

D. The use of strong native promoters

Efficient synthesis of recombinant proteins in filamentous fungi is achieved by placing the target gene under the control of a promoter mediating strong transcription. The term promoter used here refers to the sequences upstream of the translation start codon and may include sequences to which regulatory proteins bind. Fungal promoters which have been used for the expression of homologous or heterologous genes can be divided into two groups: promoters from constitutive or inducible, highly expressed genes. The most frequently used constitutive promoter is from gene encoding glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) in *A. nidulans*, which was successfully used for the production of human interleukin-6 (Broekhuijsen *et al.*, 1993), and hen egg-white lysozyme (HEWL) (Archer *et al.*, 1990a) by *A. niger* and also shown to be functional in another species, *Penicillium chrysogenum* (Kolar *et al.*, 1988). On

the other hand, the most frequently used inducible promoter in *A. niger* is the glucoamylase (P_{glaA}) promoter, which is starch-inducible (Archer and Peberdy, 1997). Another common fungal promoter is the cellulose-inducible promoter of the cellobiohydrolase (*cbh1*) gene from *T. reesei*. Table 1.4 lists some examples where P_{glaA} and P_{cbh1} promoters have been successfully used for secreted protein production, although many other fungal promoters have been successfully used for secreted protein production, but not listed in Table 1.4 because they are not directly related to this study.

Table 1.4 Examples of using P_{glaA} and P_{cbh1} for secreted protein production

Host strain	Promoter (origin of secretion signal sequence)	Heterologous protein	Reference
<i>A. niger</i>	<i>GlaA</i> (<i>A. niger</i>)	Hen egg white lysozyme	(Archer <i>et al.</i> , 1990a)
<i>A. niger</i>	<i>GlaA</i> (<i>A. niger</i>)	Bovine enterokinase	(Svetina <i>et al.</i> , 2000)
<i>A. niger</i>	<i>GlaA</i> (<i>A. niger</i>)	Fragments scFv antibody	(Hessing <i>et al.</i> , 1996)
<i>A. niger</i>	<i>GlaA</i> (<i>A. niger</i>)	Bovine prochymosin	(Ward <i>et al.</i> , 1990)
<i>A. niger</i>	<i>GlaA</i> (<i>A. niger</i>)	Aspartic proteinase	(Ward <i>et al.</i> , 1993)
<i>A. oryzae</i>	<i>GlaA</i> (<i>A. oryzae</i>)	Chymosin	(Tsuchiya <i>et al.</i> , 1993)
<i>A. oryzae</i>	<i>GlaA</i> (<i>A. niger</i>)	Lactoferrin	(Ward <i>et al.</i> , 1995)
<i>A. nidulans</i>	<i>GlaA</i> (<i>A. niger</i>)	Interleukin-6	(Contreras <i>et al.</i> , 1991)
<i>T. reesei</i>	<i>Cbh1</i> (<i>T. reesei</i>)	Xylanase	(Paloheimo <i>et al.</i> , 2003)
<i>T. reesei</i>	<i>Cbh1</i> (<i>T. reesei</i>)	Fab antibody fragments	(Nyyssönen and Keränen, 1995)
<i>T. reesei</i>	<i>Cbh1</i> (<i>T. reesei</i>)	Xylanase	(de Faria <i>et al.</i> , 2002)

A study with *A. niger* has shown that the expression of a heterologous gene, encoding the bacterial *Vitreoscilla* haemoglobin (VHb) (Dikshit and Webster, 1988), was improved by introducing multiple copies of a protein-binding sequence containing CCAAT (a 38 nt region designated as region-I) to the P_{glaA} (Liu *et al.*,

2003). DNAase I footprinting studies have shown a protected region in the *P_{glaA}* that contains a CCAAT sequence (Qiu *et al.*, 2002). It has been reported for many genes in higher eukaryotes as well as some genes in filamentous fungi that the CCAAT motif is essential for high level expression (Brakhage *et al.*, 1999; Kato *et al.*, 1997; Tani *et al.*, 2001b; Tani *et al.*, 2001a). Minetoki *et al.* (1998) reported that the introduction of multiple copies of a conserved region designated region-III into the *agdA* (encoding α -glucosidase of *A. oryzae*) promoter results in overexpression of α -glucosidase (Minetoki *et al.*, 1998). Comparison of region-I of *P_{glaA}* and region-III of *agdA* showed no significant homology except for the CCAAT motif, suggesting a critical role for the CCAAT motif in the transcription of the starch hydrolysis genes in *Aspergillus* (Liu *et al.*, 2003). However, whether the flanking sequences of these regions have a role in the expression of their downstream gene is not known.

A better understanding of fungal promoters as well as the transcription factor(s) that mediates the regulation of the promoter should provide useful information to improve the process of protein secretion in filamentous fungi.

1.2 Secretion pathway

The molecular mechanisms of the process of protein secretion have been studied more extensively in yeast and mammalian systems than in filamentous fungi. For this reason in this Chapter, the secretory pathways as known in yeast will be described in more detail than in filamentous fungi; furthermore, comparison with mammalian systems will only be used when useful. Although there are significant morphological and physiological differences between filamentous fungi and yeast or higher eukaryotes, several components of the secretion machinery are conserved in eukaryotes (van Gorcom *et al.*, 1994). Furthermore, a number of filamentous fungal homologues of yeast and higher eukaryotes genes involved in the process of protein secretion have been identified (Sakaguchi, 1997; van Gorcom *et al.*, 1994).

1.2.1 Endoplasmic reticulum (ER) targeting

Targeting of secretory proteins to the ER is the first committed step in the secretory pathway. Generally, all eukaryotic proteins destined for secretion contain an ER targeting signal known as the signal sequence, signal peptide, or leader sequence as an amino-terminal extension of the secreted protein. Signal peptides consist of three regions: an amino-terminal region (N-region) with the most positively charged amino acid residues, a central hydrophobic region (H-region) consisting of hydrophobic amino acid residues, and a polar carboxyl-terminal region (C-region) that includes the signal sequence cleavage site (von Heijne, 1990). The signal sequence is recognized by a cytosolic ribonucleoprotein complex termed signal recognition particle (SRP). The function of the SRP is to mediate the translocation of the nascent polypeptide-ribosome complex to the ER membrane and this process will be discussed later in this section. It has been suggested, using *in vitro* translation-translocation, that not only do the properties of each region of the signal sequence individually determine its topogenic function, but the properties of one region can affect the topogenic function of another region. For instance, varying the length of the hydrophobic segments showed different topogenic functions for that region: for example, longer hydrophobic region are often associated signal anchor sequences. Thus it was proposed that the function of N-terminal topogenic sequences depends on a balance between the N-terminal charge and the length of the following hydrophobic segment (Sakaguchi *et al.*, 1992). Also the hydrophobicity of the H-region determines the efficiency of the signal peptide function and thus the hydrophobicity of this region is related to the efficiency of directing the protein translocation (Bird *et al.*, 1990). The last region (C-region) contains the sequence that is recognised by the signal peptidase complex (SPC). In yeast the SPC consists of four different polypeptides, namely Spc1p, Spc2p, Spc3p and Sec11p. The SPC complex cleaves the signal sequence from the pre-protein (Meyer and Hartmann, 1997; YaDeau *et al.*, 1991). Some secretory proteins contain, in addition to the pre-sequence, a pro-sequence. However, the pro-sequence is removed in the late

Golgi by the endoprotease Kex2p, which cleaves the pro-sequence at the dibasic sequence (Mizuno *et al.*, 1989).

In eukaryotes there are two pathways by which proteins are targeted and transported across the ER-membrane or integrated into it. These pathways include the SRP-dependent pathway in which proteins are targeted to the ER co-translationally and the SRP-independent pathway in which proteins are targeted to the ER post-translationally (Figure 1.1) (Rapoport *et al.*, 1996; Sakaguchi, 1997; Stirling, 1999; Walter and Johnson, 1994).

In the co-translational pathway, the signal sequence of the growing peptide is recognised by the SRP and elongation is either arrested or slowed down by the SRP. The next step in the SRP-dependent targeting route to the ER is that the whole complex consisting of the ribosome, the nascent chain, and the SRP binds to the ER membrane. The binding of this complex to the ER membrane involves two different interactions. One occurs between the SRP and its ER membrane receptor and the other occurs between the ribosome and the ER membrane proteins. Once these interactions have taken place, the SRP is released by GTP-hydrolysis on the SRP54 subunit (Rapoport *et al.*, 1996). The release of SRP allows the interaction between the ribosome/nascent chain with Sec61 protein complex which mediates the translocation process (Stirling, 1999).

After targeting, polypeptides are translocated through the ER membrane at specific translocation sites called translocons. Translocons are heterotrimers of the membrane proteins comprising the Sec61 complex. The mammalian homologue of the Sec61 of *S. cerevisiae* is Sec61 α which shows amino acid identity of 56% to that of the yeast protein (Gorlich *et al.*, 1992a). In yeast, the Sec61 complex is a trimeric complex consisting of Sec61p, Sss1p, and Sbh1p. Interestingly, some corresponding components of the mammalian translocon show high sequence similarity to those of yeast. For instance, the smallest-sized subunit in yeast and mammalian translocon, Sss1p and Sec61 γ , respectively, are highly related and Sec61 γ can replace Sss1p in yeast cells (Hartmann *et al.*, 1994).

Co-translational targeting and translocation

Post-translational targeting and translocation

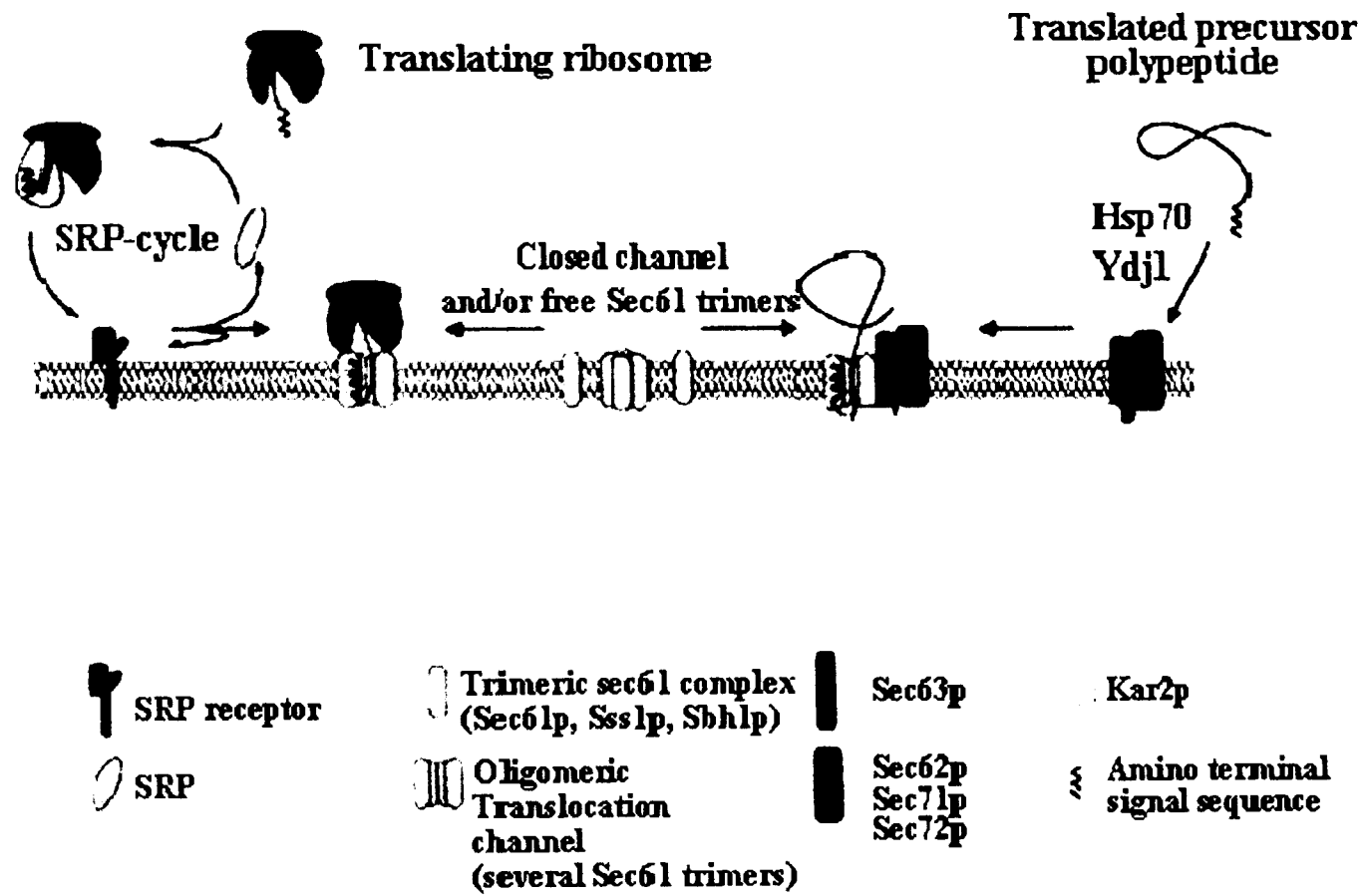


Figure 1.1 Protein translocation across the ER membrane in yeast. (Figure was adapted from Stirling, 1999).

The intermediate-sized subunits of the trimeric complex in yeast and mammalian Sbh1p and sec61 β , respectively, are clearly related (Panzner *et al.*, 1995). However, in mammalian systems an additional component may be involved in the translocation process. This component is an integral membrane protein known as translocating protein associating membrane (TRAM) (Gorlich and Rapoport, 1993). TRAM, a glycoprotein that has 6 or 8 predicted membrane-spanning segments and a cytoplasmic C-terminal tail of about 60 residues, has been shown to be involved in *in vitro* translocation of several proteins (Gorlich *et al.*, 1992a; Voigt *et al.*, 1996). No homologue of TRAM is found in yeast (Stirling, 1999).

The other pathway of protein targeting and translocation is the SRP-independent pathway. One suggested factor by which the translocation pathway is chosen is the hydrophobicity of the H-region of the signal sequence. If the binding between the signal sequence and the SRP occurs with high affinity, proteins with a less hydrophobic signal sequences are targeted via the post-translation pathway, whereas both routes can be followed when a more hydrophobic signals are present (Ng *et al.*, 1996).

In the post-translational pathway (Figure 1.1), which occurs independently of the SRP-receptor interaction, the cytosolic heat shock protein 70 (Hsp70) and Ydj1, a DnaJ homologue (Cyr *et al.*, 1992), maintain the precursors in a translocation-competent conformation (Deshaies *et al.*, 1988). Precursors in association with a membrane protein complex comprising Sec62p, Sec63p, Sec71p and Sec72p are first targeted to the Sec63 complex. Next, the whole complex is targeted to the Sec61 complex which facilitates translocation. The Sec63 complex in this post-translational pathway is likely to be involved in two processes. Firstly it targets the precursor in a similar way to the SPR receptor and, secondly, it recruits a luminal Kar2p and Lhs1p to the inner surface of the translocation site. The Kar2p is thought to drive the translocation of the pre-protein by sequential rounds of binding and release that 'pull' the pre-protein to the ER lumen (Stirling, 1999).

The possibility that in filamentous fungi similar translocation pathways to those in yeast are found is supported by the findings of a homologue of the SRP

protein SRP54 and Kar2p (BipA in *A. niger*) to those of *S. cerevisiae* (Thompson *et al.*, 1995; van Gemeren *et al.*, 1997).

1.2.2 Protein modifications in the ER

I. ER lumen

The ER lumen provides a suitable environment where newly translocated polypeptides can be folded, modified, and glycosylated before they exit to the Golgi. Some of these modification processes in the ER are assisted *in vivo* by proteins known as molecular chaperones and foldases. It is not only the presence of these ER-resident proteins that makes the environment of the ER lumen suitable for protein maturation, but also the presence of other components is required for the function of the ER. For instance, the high luminal concentration of Ca^{2+} is required for the maturation of many proteins, such as antichymotrypsin (Lodish and Kong, 1990) and, in higher eukaryotes, Ca^{2+} is a key component of the calreticulin/calnexin cycle which is responsible for the folding of newly synthesized glycoproteins and plays a role in the ER quality control (Michalak *et al.*, 2002). Furthermore, the highly oxidising environment promotes the formation of disulphide bonds (Woycechowsky and Raines, 2000) which are common in many secreted proteins. Finally, the presence of other components in the lumen, such as ATP (adenosine 5'-triphosphate), is required for the function of the luminal proteins that assist in protein folding, like the molecular chaperone Bip which depends on ATP for its function (Helenius *et al.*, 1992). In this section various components of the ER will be described including foldases, chaperones, and the interaction between these components.

A. Foldases

Genes encoding PdiA/Pdi1 (protein disulphide isomerase) have been isolated and characterised from *A. niger* and *T. reesei* respectively (Ngiam, 1998; Ngiam *et al.*, 1997; Saloheimo *et al.*, 1999). Pdi is a protein thiol-oxidoreductase that catalyses the formation, and isomerisation of protein disulphide bonds (Noiva,

1999). In filamentous fungi, some *pdi*-related genes have been identified. These genes that have been isolated include *tigA*, *erp38*, and *prpA* in *A. niger*, *Neurospora crassa* and *A. niger* var. *awamori* respectively (Jeenes *et al.*, 1997; Wang and Ward, 2000). TigA and Erp38 show 66% identity at the amino acid level, and both show a similarity of 58% and 61%, respectively, to the product of a stress-inducible gene, G1, from the alfalfa plant (*Medicago sativa* L) (Shorrosh and Dixon, 1992). Similarity is defined using a reference matrix file compiled from multiple sequence alignments which considers both physicochemical similarity and the frequency of mutation between one amino acid and another (Jeenes *et al.*, 1997). Like many ER-resident proteins, TigA is up-regulated (2-3 fold) as a result of tunicamycin treatment (Ngiam *et al.*, 2000) which disrupts ER function by inhibiting N-linked glycosylation (Dorner *et al.*, 1990; Shorrosh and Dixon, 1992). The other *pdiA* related gene isolated from *A. niger* var. *awamori*, *prpA* (Wang and Ward, 2000), contains only a single conserved thioredoxin domain near to the N-terminus of the protein. Northern hybridization showed that the transcript level of the *prpA* gene was increased in transformants secreting a heterologous protein, bovine prochymosin. However, over-expression of the *prpA* gene was not enough to improve the yields of chymosin, whereas its deletion reduced the yield of bovine chymosin by up to 80%. Furthermore, *prpA* or *tigA* are not essential for viability (Wang and Ward, 2000), suggesting some degree of functional overlap between PdiA family proteins (TigA, PrpA and PdiA). Reduction of PdiA levels by antisense RNA in *A. niger* reduced glucoamylase (GlaA) secretion by 70% (Ngiam *et al.*, 2000). However, the impact of varying the level of PdiA in *A. niger* transformants expressing a heterologous protein, thaumatin, was examined (Moralejo *et al.*, 2001). This was done by introducing additional copies of the *pdiA* gene, and transformants with different levels of *pdiA* mRNA and measured PdiA levels were examined. In addition, the secretion of two native proteins, α -amylase and acid phosphatase, were also examined in these transformants. It was found that over a range of PdiA levels of 1-8, relative to native levels in the parent strain with only one copy of the *pdiA* gene, the level of the native proteins, α -amylase and acid phosphatase, in the total secreted protein was unaffected. In contrast, the level of

thaumatin was increased about 5-fold in the transformants with PdiA levels of 3–4 fold of that of the parental strain with only a single copy of *pdiA*. The observation that changing PdiA levels affected the level of the heterologous protein, thaumatin, but not the level of the examined endogenous extracellular enzymes might be explained by the different numbers of disulphide bonds in each of the proteins. More specifically, thaumatin has eight disulfide bonds, whereas none or a small number of disulphide bonds are present in the endogenous α -amylase or acid phosphatase (Moralejo *et al.*, 2001). In *S. cerevisiae*, enhanced levels of Pdi1p have improved the secretion of disulphide bond-rich proteins such as antistasine (Schultz *et al.*, 1994) or the platelet-derived human growth factor (Robinson *et al.*, 1994). Furthermore, in yeast transformants with higher levels of PdiA, 5.4–7.9 fold of that of the parental strain, lower levels of thaumatin were secreted. It is not known precisely why lower levels of thaumatin were secreted – it was suggested that high levels of PdiA modified the folding equilibrium and led to the synthesis of non-secretable forms of thaumatin; also, the PdiA has a chaperone activity which at high levels could promote the aggregation of immature proteins (Moralejo *et al.*, 2001). However, these observations indicate that the structure of the heterologous protein plays a critical role in determining the efficiency of its secretion from the fungus strain.

In both mammalian and yeast systems PDI-like proteins exist (Chaudhuri *et al.*, 1992; Frand and Kaiser, 1998; Pollard *et al.*, 1998; Srivastava *et al.*, 1991). Generally, these proteins resemble PDI in size and modular organization. In *S. cerevisiae* Eug1p is another protein disulphide isomerase of the endoplasmic reticulum lumen that functionally overlaps with Pdi1p. Only the double mutant $\Delta pdi1 \Delta eug1$ is not viable (Tachibana and Stevens, 1992). The promoter regions of both genes encoding Pdi1p and Eug1p contain an unfolded protein response element (UPRE), suggesting the possibility that *EUG1*, like *PDI1* (Shamu *et al.*, 1994), is also up-regulated in the presence of unfolded proteins in the ER. Furthermore, *ERO1* is another gene in *S. cerevisiae* that is required for disulphide-bond formation. Interestingly, overexpression of *ERO1* confers resistance to dithiothreitol (DTT) whereas mutation of *ERO1* results in more hypersensitivity to

DTT (Frand and Kaiser, 1998; Pollard *et al.*, 1998). It was proposed that glutathione, which is known to function as a redox buffer to maintain oxidising conditions in the ER, is not required for folding and it was also concluded that Ero1p sustains the ER oxidizing potential by either oxidizing the secreted proteins or members of the PDI family, which may then act as oxidants (Frand and Kaiser, 1998; Zapun *et al.*, 1999).

On the other hand, in mammalian systems, ERp57, similar to PDI, catalyses disulphide-bond rearrangements during the refolding of ribonuclease B. However, ERp57 catalyses the disulphide-bond rearrangement of its substrate efficiently only in the presence of calnexin or calreticulin (Zapun *et al.*, 1998). Thus, ERp57 is specifically involved in the folding of glycoproteins which are bound to calnexin or calreticulin.

B. Binding Protein (Bip)

Bip is a molecular chaperone that belongs to the Hsp70 family. Molecular chaperones are defined as a functional class of families of protein that assists the correct non-covalent assembly of other polypeptide-containing structures *in vivo*, but are not components of the final assembled structures (Ellis, 1993). Bip was originally identified in mammalian cells as immunoglobulin heavy chain binding protein (Rose *et al.*, 1989) and the glucose regulated protein, GRP78 (Lee 1992). Protein chaperones such as Bip do not actively catalyze protein folding, but rather maintain proteins in a folding-competent state and prevent protein aggregation (Shen *et al.*, 2004). Bip is involved in a number of processes related to protein biogenesis (Mayer and Bukau, 1998), including the translocation of nascent polypeptides into the ER, protein folding and assembly in the ER, and degradation of proteins that could not reach their proper conformation. Some of these functions may require the involvement of co-chaperones, as in the case of maintaining the polypeptide in a retro-translocation-competent state for degradation (Kabani *et al.*, 2003). Bip hydrolyses ATP concomitant with the binding of peptides with overall hydrophobic character (Flynn *et al.*, 1991). In yeast, it has been previously proposed that Kar2p associates with Ire1p to repress

the activation of Ire1p in non-stressed cells, and in response to accumulation of unfolded proteins in the ER, Kar2p dissociates from Ire1p, resulting in dimerization and activation of Ire1p (Kimata *et al.*, 2003). Recently an *in vivo* mutation study of the yeast *S. cerevisiae* *IRE1* gene suggested that Bip (Kar2p) dissociation from Ire1p seemed to require the function of Ire1p sub-domains that are structurally independent of the Bip-binding site (Kimata *et al.*, 2004). In that study two sub-regions in the Ire1 were proposed to constitute the core stress-sensing machinery, which, in response to ER stress, become activated to dimerise, and release Bip from Ire1. Unexpectedly, mutations that abolished binding of Bip did not affect Ire1 activity under ER-stress triggered by tunicamycin or DTT treatment. However, it was proposed that the stress sensing machinery by Ire1p sub-domains was insufficient, and Bip functions as an adjustor to limit Ire1 activation to respond to ER stress (Kimata *et al.*, 2004). More studies are needed to elucidate the role of Bip under ER stress.

The gene encoding BipA has been cloned from *A. niger* based on the high sequence similarity with the *S. cerevisiae* *KAR2* gene (van Gemeren *et al.*, 1997). Furthermore, both *bipA* and *KAR2* are essential for viability. However, a correlation between *bipA* induction and secretion is unclear. For instance, up-regulation of *bipA* has been observed from *A. niger* overproducing several homologous or heterologous proteins (Ngiam *et al.*, 2000; Punt *et al.*, 1998), whereas *bipA* remained unchanged when the heterologous gene encoding interleukin-6 was expressed (Punt *et al.*, 1998). These data may suggest that overexpression of some proteins may lead to increased levels of unfolded proteins which then caused *bipA* overexpression, since *bipA* in *A. niger*, as in other organisms, is overexpressed in situations of cellular stress such as glucose starvation, heat shock, and growth conditions that lead to UPR activation (i. e. DTT treatment) (Mori *et al.*, 2000b; Ngiam *et al.*, 2000; van Gemeren *et al.*, 1997).

Finally, studies on the folding of glycoproteins have emphasised that there is a sequential recruitment of chaperones by the nascent polypeptide chain (Molinari and Helenius, 2000).

C. Calnexin and Calreticulin

Calnexin (membrane-bound) and calreticulin (the soluble homologue of calnexin, which appears to be restricted to higher eukaryotes) are calcium-binding lectin-like chaperones. They specifically interact with partially-trimmed monoglucosylated N-linked oligosaccharides and are an important part of the ER quality control. Recently, the calnexin gene has been cloned from *A. niger* (Wang *et al.*, 2003), and they showed that the steady-state levels of *clxA* mRNA were elevated in a strain overproducing a secreted heterologous protein (bovine prochymosin). Overexpression of *clxA* resulted in a four- to five-fold increase in the extracellular manganese peroxidase (MnP) levels (Conesa *et al.*, 2002). However, a comparison of glucoamylase and α -amylase levels between a calnexin disruptant and the wild-type parent by SDS-PAGE showed no detectable differences, suggesting that levels of secreted proteins remain unaffected in the *clxA* deletion strain (Wang *et al.*, 2003). Furthermore, *clxA* is not an essential gene in *A. niger* and mammalian cells whereas in *Schizosaccharomyces pombe* the gene is essential for viability (Jannatipour and Rokeach, 1995). In contrast, co-overexpression of calnexin (together with calreticulin and Bip) did not increase the formation of hepatitis C virus envelope protein complexes in mammalian cells (Choukhi *et al.*, 1998). On the other hand, calnexin disruption in *S. cerevisiae* resulted in a 2.5-fold increase in the secretion of unstable lysozyme mutants (Arima *et al.*, 1998). So this variation in calnexin's role in the improvement of heterologous protein production suggests that different heterologous proteins require different chaperones for their improved secretion.

II. ER quality control (ER-QC)

Information about the ER-QC that is discussed in this section was generated from studies carried out mainly on mammalian and yeast systems. The ER quality control in the cell allows only correctly folded proteins to proceed to their final destination. In general, the main ER-QC strategies include retention in the ER, ER-associated degradation (ERAD), retrieval to the ER from downstream

organelles, and re-routing from the Golgi complex to lysosomes or vacuoles (Ellgaard *et al.*, 1999). Although some components of the ER discussed in this section are discussed elsewhere in this Chapter, they will be discussed below from the perspective of their role in the ER-QC. The role of the ER-QC is essential for several reasons, one of which is that, by preventing the exit of premature or misfolded protein from the ER, more exposure time is allowed to the folding machinery in the ER lumen, thus offering a better chance for correct folding. This is a crucial checkpoint because the downstream organelles in the secretory pathway do not generally support protein folding (Mezzacasa and Helenius, 2002). Also, the delivery of incompletely folded proteins to their final destination could be toxic for the cell. For example, it is essential that non-functional or partially functional ion channels, transporters, and receptors do not reach the plasma membrane, where their presence could be toxic (Bichet *et al.*, 2000; Zerangue *et al.*, 1999).

Generally, two levels of quality control in the ER can be seen, 'primary quality control' that is applied to all proteins and 'secondary quality control' that operates at a specific level and is applied to selected categories of proteins (Ellgaard and Helenius, 2003; Ellgaard *et al.*, 1999). In mammalian cells, molecular chaperones and foldases involved in the primary quality control include BIp, calnexin, calreticulin, glucose-regulated protein (GRP) 94, PDI and ERp57. The recognition of incompletely folded proteins by these primary control factors is based on general biophysical properties shared by these incompletely folded proteins; these include the presence of hydrophobic surface patches, mobile loops, and a lack of compactness (Ellgaard *et al.*, 1999). Incompletely folded proteins are bound to one or more of these factors and this interaction would, in addition to assisting in the folding and assembly process, result in the retention of these incompletely folded proteins in the ER (Helenius *et al.*, 1997; Hellman *et al.*, 1999). Mutations in some endogenous proteins that prevent their proper folding and result in their accumulation in the ER could result in serious diseases (Aridor and Balch, 1999; Rutishauser and Spiess, 2002). Since no specific motif is needed for primary quality control, this should apply to heterologous proteins as

well. A well-characterised part of the primary quality control system is the calnexin/calreticulin cycle. The calnexin/calreticulin cycle ensures the correct folding of glycoproteins, and retains incorrectly folded proteins in the ER until they are correctly folded or targeted for degradation (Ou *et al.*, 1993; Ware *et al.*, 1995).

The secondary quality control involves selective mechanisms that regulate the export of specific individual proteins or protein families. For example, in yeast Shr3p is a non-essential ER-resident membrane protein that is specifically required for delivery of amino acid permeases to the plasma membrane (Herrmann *et al.*, 1999). In wild-type cells newly synthesized cargo proteins, including the general amino acid permease (Gap1p), are associated with the coat proteins (COPII) that form the transport vesicles from the ER to the Golgi compartment. However, in *shr3* null mutants, Gap1p fails to be packed into the COPII transport vesicles and accumulates in the ER, but other cargo proteins are unaffected (Herrmann *et al.*, 1999).

Another important strategy in the ER-QC is the ER-retention and -retrieval signal. These signals are important for the localization of some ER resident proteins, since continued importing and exporting processes in and out of the ER are active (Harter and Wieland, 1996; Teasdale and Jackson, 1996). For example, a well characterised retrieval sequence present at the carboxyl terminus of many soluble ER proteins is Lys-Asp-Glu-Leu (KDEL) in animal cells (Munro and Pelham, 1987) or His-Asp-Glu-Leu (HDEL) in *S. cerevisiae* (Pelham, 2000) that interacts with its receptor, a 26 kD integral membrane protein encoded by the *ERD2* gene in *S. cerevisiae* (Semenza *et al.*, 1990), and ensures that the protein is retrieved from the Golgi complex by coatamer protein (COPI) -coated vesicles (Munro and Pelham, 1987; Pelham, 2000). More examples of protein-specific QC factors are listed in Table 1.5, but will not be discussed in detail, since their discussion is not directly related to the aims of this study.

The ER-Associated Degradation (ERAD) mechanism plays an important role in the ER-QC. ERAD is carried out by the 26S proteasome located in the cytosol. Inhibition of proteasome activity in mammalian cells results in retention of a misfolded α_1 -antitrypsin, a monomeric glycoprotein member of the serine

proteinase inhibitor superfamily, and leads to a stable association with calnexin (Liu *et al.*, 1999). This suggests that calnexin is an active participant in the proteasomal degradation of misfolded α_1 -antitrypsin. It was suggested that this also implies that the central role of the enzyme uridine 5'-diphosphate (UDP) - glucose:glycoprotein glucosyltransferase (GT), which catalyses the first step in the synthesis of lipid-linked oligosaccharides, as a folding sensor is important not only in the calnexin/calreticulin cycle, but also for targeting of misfolded protein for proteasomal degradation (Ellgaard *et al.*, 1999).

Table 1.5 Examples of protein-specific QC factors and their target substrates and functions

^a Abbreviations:

Chs	chitin synthesis
Dgt	delayed GPI-anchored protein transport
GPI	glycosyl phosphatidylinositol-linked protein
HSP	heat shock protein
LST	lethal with sec-thirteen
Lag	longevity-assurance gene
MHC	major histocompatibility complex
RAP	receptor-associated protein
RIC	resistant to inhibitions of cholinesterase

Factor	Organism of cell type	Substrate	Function	Reference
LST1 ^a	<i>S. cerevisiae</i>	Plasma membrane H ⁺ -ATPase Pma1p	Potential cargo receptor	(Roberg <i>et al.</i> , 1999)
Erv14p (ER-vesicle protein of 14 kD)	<i>S. cerevisiae</i>	Plasma membrane protein Axl2p	Potential cargo receptor	(Powers and Barlowe, 1998)
Chs7p ^a (involved in chitin synthesis)	<i>S. cerevisiae</i>	Chs3p catalytic subunit of chitin synthetase III	Ensures ER secretion competence	(Trilla <i>et al.</i> , 1999)
Lag1p ^a and Dgtp1 ^a (ER transmembrane proteins)	<i>S. cerevisiae</i>	GPI-anchored proteins Gas1 and Yap3p	Facilitates ER secretion of GPI-anchored proteins	(Barz and Walter, 1999)
P24 family (23-to27-kD type I transmembrane proteins)	<i>S. cerevisiae</i> Mammalian cells	Various secreted proteins	Potential cargo receptor for a subset of secretory and membrane proteins	(Marzioch <i>et al.</i> , 1999)
HSP47	Mammalian collagen-producing cells	Procollagen	Specialised collagen chaperone	(Nagata, 1998)
RAP ^a	Mammalian cells	LDL receptor family and other transmembrane receptor	Prevents aggregation and premature ligand binding	(Bu <i>et al.</i> , 1995)
Tapasin	Mammalian cells	MHC class I	Prevents ER exit to MHC class I without bound antigenic peptide	(Ortmann <i>et al.</i> , 1997)

1.3 The Unfolded Protein Response (UPR)

Perturbation of the protein folding in the ER can be triggered by several conditions. These conditions include: the use of chemical agents such as DTT, tunicamycin, the brefeldin A (BFA), the ionophore A23187 and expressing mutant secretory proteins that do not fold properly. These ER perturbation conditions result in accumulation of unfolded or misfolded proteins in the ER. The cell can sense the presence of the unfolded proteins in the ER and respond to changes in the normal functioning of this specialised compartment for protein folding and processing. A sensing mechanism that enables the cell to determine the time a protein has spent attempting to fold and thus allowing the cell to initiate the stress response which may redirect the problem protein for degradation should be present (MacKenzie *et al.*, 2004). In mammalian cells, several studies have suggested that the demannosylation of the core glycan from a Man9 to Man8 structure by ER mannosidase I is part of this timing mechanism (Fagioli and Sitia, 2001; Liu *et al.*, 1999). The inhibition of protein folding within the ER leads to cellular responses, collectively called ER stress, and the UPR is the most common cellular response that has been studied extensively in different eukaryotes, such as in mammalian cells *S. cerevisiae*, *Arabidopsis thaliana*, and filamentous fungi (Cox and Walter, 1996; Dorner *et al.*, 1990; Lee, 1992; Martinez and Chrispeels, 2003; Mori, 2000a; Mulder *et al.*, 2004; Saloheimo *et al.*, 2003). Genomic analysis studies under active UPR showed that, rather than regulating the level of only ER-resident chaperones and foldases, as previously thought, the UPR affects the regulation of genes involved in nearly all stages of the secretory pathway (Benedetti *et al.*, 2000; Martinez and Chrispeels, 2003; Travers *et al.*, 2000). UPR is also essential for ERAD, and although the sensor involved in ERAD is not known, research suggests that the UPR and ERAD are very closely integrated. Gene expression analysis studies reveal an intimate coordination between these responses: efficient ERAD requires an intact UPR, and UPR induction increases ERAD capacity. On the other hand, loss of ERAD leads to constitutive UPR induction (Travers *et al.*, 2000). ER stress in mammalian cells leads to

phosphorylation of the eukaryotic translation initiation factor 2 α which down-regulates translation of secretory proteins but this effect has not been reported in yeasts or filamentous fungi. All these diverse feedback mechanisms are believed to provide adaptive responses for survival, since failing to correct protein-folding defects leads to cell apoptosis (Travers *et al.*, 2000). In the following sections the feedback mechanisms activated in response to ER stress known collectively as UPR will be discussed mainly in *S. cerevisiae* and filamentous fungi systems. However, a comparison with mammalian systems will be used when believed to be useful.

1.3.1 Activation of the UPR mechanism in *S. cerevisiae*

The UPR mechanism has been studied in *S. cerevisiae* more extensively than in other microorganisms (Figure 1.2). The main components of the UPR identified to date that mediate this mechanism include the following proteins: Ire1p, Rlg1p, Hac1p, and Ptc2p. The Ire1p is a transmembrane kinase that transmits the signal across the ER or inner nuclear membrane. The transmembrane Ire1p has both kinase and endoribonuclease activities (Sidrauski and Walter, 1997). The N-terminal half of the protein is in the lumen of the ER, whereas the C-terminal is in the cytoplasm (Mori *et al.*, 1993). Under normal, unstressed conditions, Kar2p associates with the luminal domain of Ire1p and keeps it in an inactive monomeric state.

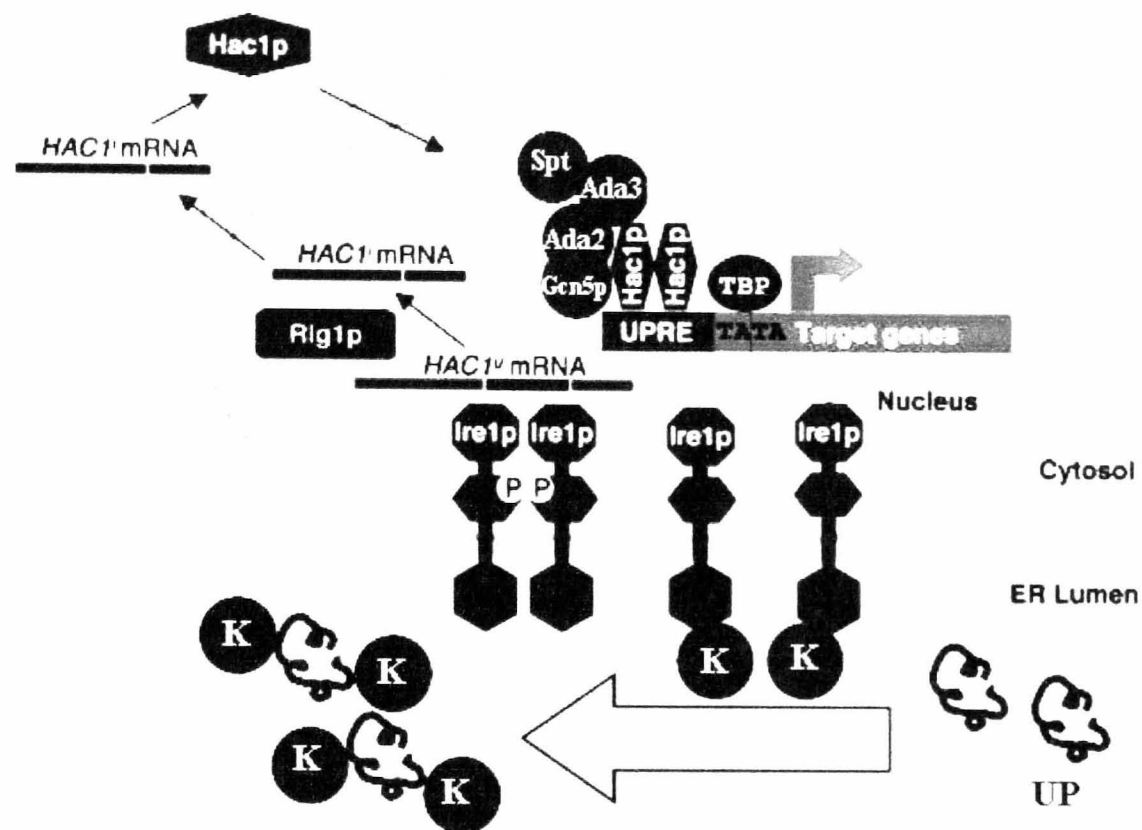


Figure 1.2 The unfolded protein response pathway in *S. cerevisiae*. UP: unfolded protein K: Kar2p, Rlg1p (tRNA ligase), Gcn5p (histone acetyltransferase), Ada2 (refers to Ada2p), Ada3 (refers to Ada3p) and spt are subunits of the SAGA transcriptional regulatory complex (adapted from Welihinda *et al.*, 2000).

Under ER stress conditions Kar2p dissociates from Ire1p, allowing it to activate by dimerisation (Shamu and Walter, 1996). The activated Ire1p trans-autophosphorylates through its cytosolic kinase domain (Bertolotti *et al.*, 2000), and this, along with the recruitment of a tRNA ligase (Sidrauski and Walter, 1997), allows the splicing of an unconventional intron from the *HAC1* precursor mRNA (Mori *et al.*, 2000b). *IRE1* is not essential under normal growth conditions, but is essential under conditions that trigger the UPR (Cox *et al.*, 1993). *Ire1* mutants are also inositol auxotrophs (Nikawa and Yamashita, 1992).

The only known substrate of the Ire1p endonuclease is the *HAC1* mRNA, which encodes the basic leucine zipper (bZIP) transcription factor. *HAC1^u* (^u for uninduced) mRNA is constitutively transcribed, but due to the presence of a non-spliceosomal intron of 252 nucleotides at the 3' end of the open reading frame (ORF), translation is blocked (Cox and Walter, 1996). The *HAC1* mRNA intron represses translation of *HAC1^u* mRNA by base pairing with the *HAC1* 5'UTR (untranslated region), which creates a stem loop structure that attenuates the translation process by ribosomes (Rüegsegger *et al.*, 2001). The removal of the 252 bp replaces a tail of 10 amino acids encoded in the intron of *HAC1^u* mRNA (encoding 230 amino acids in total) with a tail of 18 amino acids encoded in the second exon of *HAC1ⁱ* (ⁱ for induced) mRNA (238 amino acid in total). The two exons are subsequently joined by tRNA ligase to produce *HAC1ⁱ* mRNA. Kawahara *et al.* (1998) have carried out mutational analysis of *HAC1* mRNA and showed that the 5' and 3' splice sites of the nonconventional intron consist of seven nucleotides but only four of these nucleotides are specifically (even in a random order) required for the cleavage. Furthermore, swapping the nucleotide sequences of the 5' and 3' splice sites inhibited the ligation but not the cleavage step (Kawahara *et al.*, 1998). These results showed that the cleavage is sequence-specific and not sequential.

Mammalian UPR seems to be more complex compared to the yeast UPR. In yeast, as stated previously, the only known ER-to-nucleus signal transduction pathway so far is the one which is mediated by Ire1p/Hac1p, whereas in mammalian UPR at least three different ER-to-nucleus signalling pathways related

to the UPR have been reported. These pathways include IRE1/XBP1 (Wang *et al.*, 1998), ATF6 (Activating Transcription Factor) (Yoshida *et al.*, 1998), and PERK pathways (Harding *et al.*, 1999) (Figure 1.3). The mammalian genome contains two homologues of yeast Ire1p: IRE α , expressed in most cells with high levels of expression and IRE β , expressed primarily in epithelial cells of the gastrointestinal tract. Overexpression of either protein is sufficient to up-regulate ER chaperones such as Bip (Wang *et al.*, 1998). Deletion of either or both *IRE* α and *IRE* β in the mouse genome did not interfere with transcriptional activation of the endogenous *BiP* gene in cultured cells (Lee *et al.*, 2002b). However, deletion of *IRE* α causes early embryonic lethality, whereas no similar observation was shown when *IRE* β was deleted, but the mice did show increased susceptibility to experimentally induced colitis, an inflammation of the colon (Bertolotti *et al.*, 2001). One substrate of the mammalian IRE1 is *XBP1*, (X-box binding protein) a gene that encodes a transcription factor, mRNA. IRE1 removes an unconventional 26-nucleotide intron from the mRNA of mouse *XBP1* (Calton *et al.*, 2002). The mouse *XBP1* 26-nucleotide intron forms a structure with two hairpin loops of seven residues held in place by short stems. The cleavage sites within these hairpins have some conservation with the corresponding *S. cerevisiae* *HAC1* mRNA structure (Calton *et al.*, 2002). *XBPI* protein binds to a *cis*-acting ERSE (Endoplasmic Reticulum Stress Response Element), consisting of a tripartite structure CCAAT-N₉-CCACG, (Yoshida *et al.*, 1998). The ERSE sequence is also present in the *XBP1* promoter, suggesting that *XBPI* up-regulates itself (Lee *et al.*, 2003). Furthermore, another *cis*-acting element ERSEII (ATTGG-N-CCACG) has been identified from the promoter of a human ER membrane protein (HERP), which is induced by the UPR (Kokame *et al.*, 2001). The role of HERP in ER stress response was investigated by a recent study which showed that HERP is a short-lived ubiquitin-like protein that improves the balance of folding capacity and protein loads in the ER and plays a crucial role for ER stress resistance in F9 embryonic carcinoma cells (Hori *et al.*, 2004).

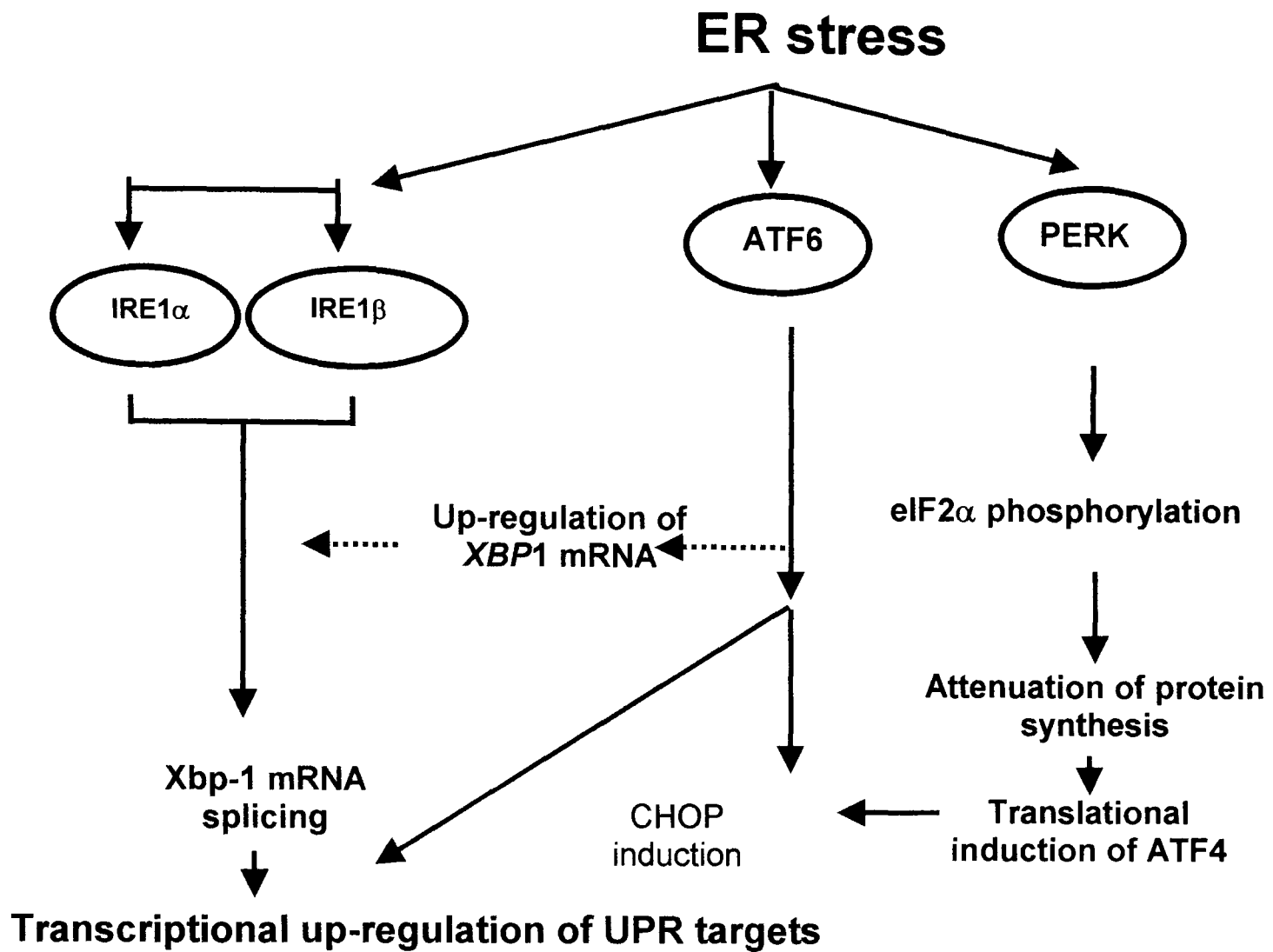


Figure 1.3 The unfolded protein response pathways in mammalian cells. Transduction of ER stress signals can be mediated via three transmembrane proteins, PERK, ATF6, and IRE1 α or β . Prolonged activation of the UPR can lead to activation of apoptosis via CHOP (Ma and Hendershot, 2001).

ATF6, a 670 amino acid ER transmembrane-activating factor, can also bind to the ERSE motif. There are two forms of ATF6: ATF6 α (90 KDa) and ATF6 β (110 KDa). Upon ER stress ATF6 α and ATF6 β transit to the Golgi compartment where they are cleaved in a two-step process by S1P and S2P (site-1 and site-2 proteases) to generate free 50-60 kDa cytosolic bZIP-containing fragments (Ye *et al.*, 2000). The free ATF6 fragments migrate to the nucleus to activate transcription of their target genes. Both forms, in the presence of the CCAAT-binding factor (NF-Y, also called CBF) bind to the 3' half-site (CCACG) of the ERSE in the promoter region of UPR-responsive genes in order to activate transcription (Haze *et al.*, 1999; Li *et al.*, 2000; Yoshida *et al.*, 2001b). More specifically, NF-Y, a trimer complex composed of three subunits, NF-YA, NF-YB, and NF-YC, binds to the CCAAT part of ERSE while ATF6 binds to the CCACG part. The separation of NF-Y and ATF6 binding sites by a spacer of 9 bp is essential for functional binding since insertion or deletion of 1 bp is sufficient to inactivate transcriptional activity of ERSE by abolishing binding of ATF6 α or ATF6 β to ERSE (Yoshida *et al.*, 2001a). Overexpression of ATF6 induces gene-encoding *XBP1*, thus ATF6 with IRE1 regulates the quantity and quality, respectively, of XBP1 protein. Moreover, ATF6 can recognise and bind to both the ERSE and ERSEII of the target genes.

In mammalian systems the UPR can be controlled at the translational level as well, a feedback response mediated mainly by the protein PERK (Harding *et al.*, 1999). PERK is an ER transmembrane protein kinase that phosphorylates the α subunit of translation initiation factor 2 (eIF2 α) in response to ER stress. Phosphorylation of eIF2 α results in down-regulation of overall protein synthesis.

Neither the existence of a functional PERK homologue nor attenuation of translation in response to ER stress has been demonstrated in filamentous fungi or yeast. In *S. cerevisiae* the Hac1ⁱ up-regulates transcription of its target genes by binding to *cis*-acting elements in their promoters, the UPR element (UPRE) which corresponds to CAGCGTG (Mori *et al.*, 1996). Mori *et al.* (1992) defined a 22 bp *cis*-acting element in the promoter of the UPR up-regulated gene encoding Kar2p. Later on, Mori *et al.* (1998) showed that the UPRE of *Kar2* and four other

UPR up-regulated genes (*PDI1*, *EUG1*, *FKB2*, and *LHS1*) contain an E box-like palindrome separated by one nucleotide (CAGCGTG) and that it was specifically recognised by Hac1p both *in vivo* and *in vitro* (Mori *et al.*, 1998). In *A. niger* it has been shown that HacA up-regulates its own transcription by binding to the promoter of the *hacA* gene itself (Mulder *et al.*, 2004). Furthermore, in *S. cerevisiae*, overexpression of the *PTC2* gene, which encodes a serine/threonine phosphatase, reduces levels of spliced *HAC1* mRNA and attenuates the UPR. Ptc2p down-regulates the UPR by dephosphorylating *Ire1p*, thus showing a mechanism of regulation in the UPR pathway upstream of the *HAC1* mRNA splicing event. Deletion of the *PTC2* gene results in increased activity of the UPR. Therefore, Ptc2p is a negative regulator of UPR signalling (Welihinda *et al.*, 1998).

Generally, in eukaryotes, transcriptional activation requires functional interaction between the co-activators that bind upstream activating sequences and the basal factors that occupy the TATA box. In yeast, it has been demonstrated that a 1.8 MDa co-activator complex named SAGA (Spt-Ada-Gcn5-Acetyltransferase) is involved in the UPR but not in the heat shock response, a stress response that up-regulates protein chaperone genes (Morano *et al.*, 1998). The Spt proteins known to be present in the SAGA complex include Spt3p, Spt7p, Spt8p, and Spt20p (Gansheroff *et al.*, 1995), and are apparently vital to the structure of SAGA, since null mutations in any of the encoding genes cause complete disruption of the SAGA complex and severe growth defects (Grant *et al.*, 1997). The importance of the SAGA complex subunits in the UPR was shown by mutation studies. Deletion of *GCN5*, *ADA2*, or *ADA3* reduces, and deletion of *ADA5* completely abolishes, the UPR (Welihinda *et al.*, 2000). Furthermore, the phenotypes that the *S. cerevisiae* $\Delta ada5$ strain showed, the UPR defect and the inositol requirement, are identical to those observed in the $\Delta ire1$ and $\Delta hac1$ strains (Cox *et al.*, 1997). Although *HAC1* mRNA requires only *Ire1p* and *Rlg1p* for activation *in vitro*, these results and the observation that *ADA5* is required for the IRE1/RLG1-dependent splicing reaction *in vivo* suggest that Ada5p may be involved in *HAC1* mRNA processing events (Welihinda *et al.*, 2000).

The UPR affects numerous genes in the secretory pathway and of the ERAD system. The UPR up-regulated gene set accounts for approximately 6% of the yeast genome (Travers *et al.*, 2000), although this does not imply a direct Hac1p-mediated activation of all such genes. Genome-wide expression analysis in conjunction with specific mutations in *S. cerevisiae* has revealed that the UPR has broader affects than previously anticipated. Travers *et al.* (2000) have combined results generated from different experiments using different stress agents to identify the set of genes involved specifically in the UPR. This was done by comparing the expression profiles under tunicamycin and DTT treatment and by using strains bearing either a deletion of *IRE1* or *HAC1*. The number of total ORFs that was induced by both DTT and tunicamycin was 381. This number may be underestimated due to the high stringency of the criteria employed for identifying these targets. Out of the 381 ORFs 208 genes have some functional information available (named genes or their homologues) and 173 genes have no information presently available. Of the functionally characterized genes, 103 are known or predicted by sequence homology to play roles in secretion or the biogenesis of secretory organelles (Travers *et al.*, 2000).

The genome-wide analysis discussed above identified 87 ORFs that are induced in response to ER stress in the strains bearing the deletion of *IRE1* and *HAC1* (Travers *et al.*, 2000). Also, it has been found that the induction of one-third of reporter constructs having UPRE- lacZ by ER stress is independent of *IRE1* and *HAC1* (Schröder *et al.*, 2003; Shamu and Walter, 1996). Schröder *et al.* (2003) have suggested the existence of an *IRE1*- and *HAC1*-independent pathway for transcriptional activation in yeast under ER stress, and proposed that this pathway culminates in stimulation of the activity of a core promoter, a process termed Core Promoter Activation (CPA) (Schröder *et al.*, 2003). In their study Schröder *et al.* (2003) isolated several mutants that allow efficient ER-to-nucleus signalling in the absence of functional *IRE1*. This was done by randomly mutating *S. cerevisiae* strains that bear deletion of *IRE1* ($\Delta ire1$) and isolated mutant strains that showed increased resistance to both tunicamycin and 2-deoxy-D-glucose (Hubbard and Ivatt, 1981), which induce accumulation of unfolded proteins in the ER lumen by

different mechanisms. All these mutant strains, which showed increased resistance to ER stress, carried a mutation in the *SIN4*-encoding gene. Sin4p is part of a mediator complex that includes Gal11p and Rgr1p, that regulates activity of the RNA polymerase II holoenzyme and is believed to be involved in positive and negative regulation of the transcription of target promoters, possibly via changes in chromatin structure (Flaus *et al.*, 2004; Li *et al.*, 1995). Mutation in *SIN4* was found to activate transcription in response to ER stress in $\Delta ire1$ strains. Two main scenarios were suggested to explain how *SIN4* mutations affect basal transcription. First, mutations in *SIN4* result in a change in the chromatin structure which increases the accessibility of the promoter for the RNA polymerase II holoenzyme (Jiang and Stillman, 1992). The change in chromatin structure could occur possibly as a result of less dense packaging of nucleosomes, increased histone acetylation, or binding of another (as yet unknown) transcriptional activator to the promoter and subsequent recruitment of the holoenzyme to the promoter. Second, mutations in *SIN4* may result in an increase in the specific activity of the holoenzyme. Examples of the second scenario could be changing the intrinsic structure of the holoenzyme such as a post-translational modification (e.g. phosphorylation or the addition or elimination of regulatory factors), which results in increased specific activity of the holoenzyme (Schröder *et al.*, 2003). Furthermore, CPA seems to selectively target specific promoters, since not all examined ER chaperones showed up-regulation. Northern analysis using the $\Delta ire1$ or $\Delta ire1 \Delta Sin41$ mutant strains showed moderate activation of some ER chaperone genes, including *KAR2*, *PDI1*, and *LHS1* (assists in protein folding and required for efficient translocation of protein precursors across the ER membrane (Hamilton and Flynn, 1996)), whereas the activation of *EUG1*, a gene-encoding a protein disulphide isomerase of the endoplasmic reticulum lumen that overlaps (in function) with Pdi1p (Norgaard *et al.*, 2001) and *SCJ1*, encoding a DNAJ homolog (Silberstein *et al.*, 1998), was completely abolished. Furthermore, those ER chaperone genes, which showed moderate activation under ER stress in the $\Delta ire1$ or $\Delta ire1 \Delta Sin41$ mutant strains showed a similar up-regulation pattern under other cellular stress responses such as heat shock or osmotic stress.

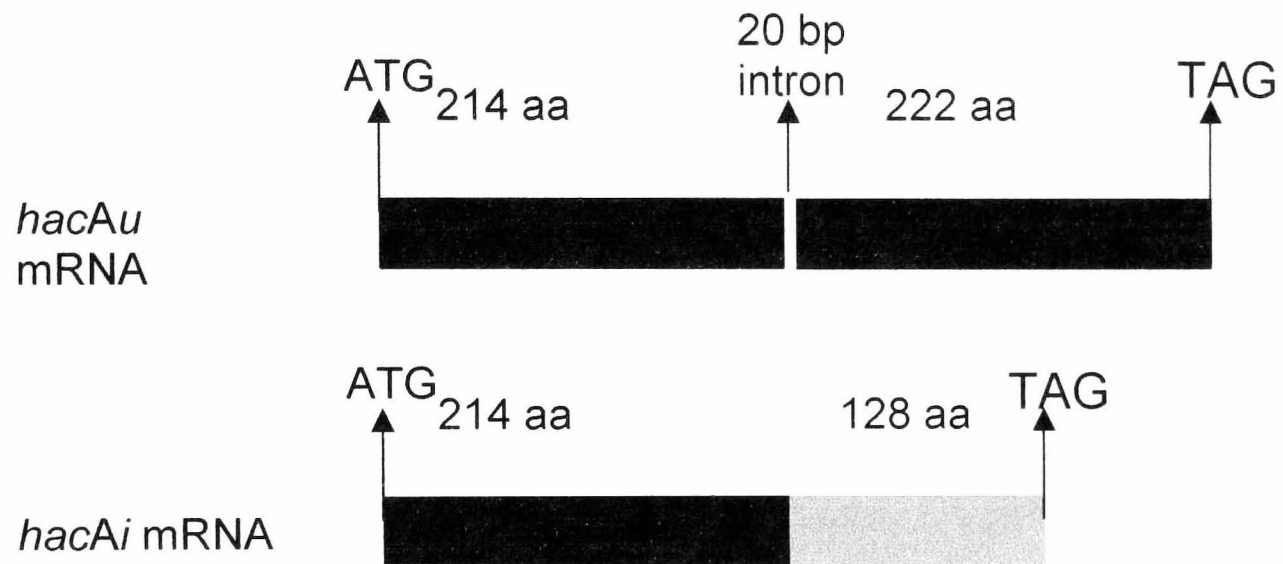
In mammalian systems it has been shown that prolonged UPR activation leads to apoptotic cell death, possibly by activating *CHOP*, a transcription factor also known as growth arrest and DNA damage gene-153 (*GADD153*) that mediates the apoptosis process (Brewer and Diehl, 2000; Wang *et al.*, 1998). Another independent apoptosis pathway that has been shown to be activated under ER stress conditions is Caspase pathway. Caspases belong to a class of cysteine proteases that includes several representatives involved in apoptosis. In brief, the caspases convey the apoptotic signal in a proteolytic cascade, with caspases cleaving and activating other caspases that then degrade other cellular targets which lead to cell death (Rao *et al.*, 2001).

1.3.2 Activation of the UPR mechanism in Filamentous fungi

The UPR signalling pathway in filamentous fungi, examined to date, appears to be analogous to that in *S. cerevisiae* (Chapman *et al.*, 1998) in so far as homologues of the membrane-bound sensor Ire1p, and the transcription factor Hac1p have been found in *T. reesei*, *A. nidulans* and *A. niger* (Mulder *et al.*, 2004; Saloheimo *et al.*, 2003). Furthermore, a homologue of Ptc2p phosphatase involved in regulating the activity Ire1p has been isolated from *T. reesei* and *A. nidulans* (M. Valkonen, M. Saloheimo, M. Penttilä, personal communication). However, maturation of the *hac* mRNA seems to be different to that reported in yeast (Kawahara *et al.*, 1997) because the filamentous fungal *hac* mRNA is truncated at the 5'-flanking region in addition to the splicing of an unconventional intron of 20-nt under ER-stress conditions (Mulder *et al.*, 2004; Saloheimo *et al.*, 2003).

Recently, the *hacA* gene was cloned from *A. niger* (Mulder *et al.*, 2004). ER stress results in the splicing of an unconventional intron of 20 nt (Figure 1.4), and is associated with truncation of the 5'-end of *hacA* mRNA by 230 nt. The gene encoding the transcription factors HacA/Hac1 have been identified in *A. nidulans* and *T. reesei*, respectively, and both have unconventional introns of 20 nt, similar

Figure 1.4 A diagram to illustrate the affect of *hacA* unconventional intron splicing on changing the ORF at the C-terminal in *A. niger*. Data used to generate this diagram were acquired from Mulder *et al.*, (2004).



The gray shaded box represents the new C-terminal of the protein following processing of *hacA* intron.

aa: amino acid

u: uninduced

i: induced

ATG: start codon

TAG: stop codon

in length to that of *A. niger*, but the truncation of the 5'-end shows variation (Saloheimo *et al.*, 2003). As detected by northern hybridisation, the *hacA* mRNA from ER-stressed mycelia was about 150 bp shorter than the *hacA* mRNA from untreated mycelia in *A. nidulans*, whereas in *T. reesei* the difference was about 250 bp. However, the site of truncation of the *T. reesei hac1* mRNA that was mapped by rapid amplification of cDNA ends (RACE)-PCR showed that the seven RACE-PCR products studied from DTT-treated samples had their 5'-ends each at a different position, ranging from 254 to 336 bp from the longest cDNA clone isolated (from untreated sample) (Saloheimo *et al.*, 2003). Recently, the site of cleavage was determined more precisely in *A. niger* (Mulder *et al.*, 2004). The truncated area from the 5'-flanking untranslated region of *T. reesei*, *A. niger*, and *A. nidulans* includes potential ORFs that is translated, would be 18, 44, and 7 amino acids, respectively (Mulder *et al.*, 2004; Saloheimo *et al.*, 2003). Also, in the remaining 5'-flanking region of the *T. reesei hac1* mRNA there is a second ORF possibly encoding two amino acids. Whether the sequence in the 5'-UTR has a translational control role is not known yet. Recently, the regulation of fungal gene expression via short open reading frames in the mRNA 5' untranslated region has been reviewed (Vilela and McCarthy, 2003). It has been shown that the expression of fungal mRNAs can be controlled by ribosome interactions with short upstream open reading frames (uORFs) within the 5' untranslated region. In *A. niger*, for example, post-transcriptional control has been proposed for the gene-encoding cytochrome P450 reductase, *cprA*, where different-sized transcripts were identified, one of which contained a short uORF (MacKenzie *et al.*, 2004; van den Brink *et al.*, 2000). In *S. cerevisiae*, *HAC1* showed elements of translational regulation by preferential association of polysomes during a nutritional shift from a fermentable to a nonfermentable carbon source (Kuhn *et al.*, 2001).

In *A. niger*, the splicing of *hacA* mRNA replaces a C-terminal region coding for 222 amino acids with a different one coding for 128 amino acids. Thus the *hacA* spliced (induced) mRNA in *A. niger* encodes for a 342 amino acid protein. Alignments of protein sequence encoded by un-spliced *hacA* mRNA from *A. niger*, *A. nidulans*, and *A. fumigatus* showed a high degree of identity (71%-75%) among

the species even in the C-terminal part downstream of the 20 bp intron, which may suggest a function for which the C-terminal is critical (Mulder *et al.*, 2004). Furthermore, the predicted secondary structure of the 20 bp intron in *A. niger*, *A. nidulans* and *T. reesei* is similar. Four different forms of *T. reesei hac1* cDNA with or without the full-length 5'-flanking region and/or the 20 nt unconventional intron were tested to complement *S. cerevisiae* where *IRE1* or *HAC1* had been disrupted. Only one *hac1* form that had both 5'-flanking region and the 20 nt intron could not complement either *HAC1* or *IRE1* disruptions (Saloheimo *et al.*, 2003). Moreover, the *hac1* form with neither 5'-flanking region nor the 20 nt unconventional intron induced the *KAR2* gene more strongly than the other forms. The intron was not removed in the *IRE1* disruptant mutant after UPR induction suggesting that *S. cerevisiae* Ire1p splices *T. reesei hac1^u* mRNA in response to UPR activation (Saloheimo *et al.*, 2003). On the other hand, in *A. niger*, overexpression of *hacAⁱ* up-regulates, in addition to chaperones and foldases, such as *bipA* and *pdiA*, the *hacA^u* (Mulder *et al.*, 2004). A similar observation was reported previously in mammalian cells where XBPI up-regulates its encoding gene (Yoshida *et al.*, 2001a).

1.4 Aim of the research and outline of the thesis

The main aim of the research reported in this thesis was to explore the transcriptional down-regulation of the *glaA* gene that occurs during ER stress in *A. niger*

Specific aims of this research were as follows:

- 1- To examine the steady-state level of the indigenous glucoamylase-encoding gene under different environmental conditions that result in activation of ER stress responses. These conditions include:
 - a- Treating *A. niger* cultures with chemical agents that interfere with protein folding (DTT, tunicamycin).
 - b- Using an *A. niger* strain with a functionally incomplete protein folding system (strain expressing antisense transcripts for the gene encoding PdiA).

- c- Using an *A. niger* strain expressing a heterologous protein (HEWL).
 - d- Varying the nitrogen concentrations in the *A. niger* growth medium.
- 2- To detect the activation of the ERAD mechanism in DTT-treated *A. niger* by detecting the transcription levels of the putative gene (*rpnG*) encoding the subunit of the 26S proteasome.
 - 3- To investigate the pathway that possibly mediates the decrease in *glaA* expression under ER stress conditions by deletion analysis of the *glaA* gene promoter using *A. niger* reporter strains.
 - 4- To study if the transcriptional down-regulation effect is a feedback mechanism that functions at the transcription level by measuring the *de novo* mRNA levels for the reporter gene by nuclear run-on experiments.

2 General Materials and Methods

2.1 Chemicals and reagents

All chemicals and reagents used were sourced from Sigma (UK), unless otherwise stated, and were of analytical reagent grade.

2.2 Strains

Aspergillus niger strains used were AB4.1 (van Hartingsveldt *et al.*, 1987), a *pyrG*⁻ strain that is auxotrophic for uridine, AS1.1 (Ngiam *et al.*, 2000), which is a multi-copy transformant of the AB4.1 strain containing an antisense *pdiA* cDNA under the control of the *glaA* promoter and the *A. nidulans trpC* terminator, and B1 which contains 10-12 copies of hen egg white lysozyme (HEWL) cDNA, encoding the mature protein, fused downstream of the DNA encoding the first 498 codons of the *A. niger* glucoamylase gene and including a KEX2-like endoproteolytic cleavage site at the fusion junction (Jeenes *et al.*, 1994). *Escherichia coli* XL1-Blue strain (Stratagene, UK) was used for both plasmid propagation and routine DNA manipulations.

2.3 Strain maintenance, media, and growth conditions

For short-term storage, *A. niger* strains were routinely maintained on potato dextrose agar (PDA) (Oxoid, UK) with a supplement of 10mM uridine for *A. niger* AB4.1. *A. niger* strains were grown at 30°C until they had sporulated (about 6-7 days), then slopes were stored at 4°C for up to 4 weeks. Slopes were made fresh for each experiment. For long-term storage, *A. niger* strains were adsorbed to silica gel and stored at 4°C. This was done by adding a fresh spore suspension, prepared by resuspending the spores from one slope in 500-1000 µl of 5% (w/v) sterilised Marvel milk, to sterile silica gel in a universal glass tube and allowed to dry for 4 days at RT. For *E. coli* strains, 50% (v/v) glycerol stocks were prepared and kept at -80°C.

Spores resuspended in 0.1% (v/v) Tween-20 were used to inoculate liquid cultures to a final concentration of 3×10^5 /ml, counted using hemocytometer, and grown in 100ml of *Aspergillus* complete medium (ACM/N/P) in 250ml conical flasks at 28°C and 150 rpm. The ACM/N/P medium contained the following: 20ml/l of ACM salts, 10ml/l ACM vitamins, 0.2% (w/v) bactopectone, 0.15% (w/v) Bacto casamino acids, 0.5% (w/v) NH_4Cl , 50 mM sodium phosphate buffer (pH 6.5), 1% (w/v) filter-sterilised carbon source of either xylose or glucose, (but starch was added directly to the liquid media before autoclaving), and 1% (w/v) uridine (if needed) (Archer *et al.*, 1990a). ACM salts contained 2.6% (w/v) KCl, 2.6% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 7.6% (w/v) KH_2PO_4 and 50ml/l trace elements solution (40mg/l $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 400mg/l $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 800mg/l $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 800mg/l $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$, 800mg/l Na_2MoO_4 , 8g/l $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$). ACM vitamins contained 400mg/l p-aminobenzoic acid, 1mg/l biotin, 100mg/l nicotinic acid, 50mg/l thiamin, 400mg/l inositol, 100mg/l riboflavin, 250mg/l pyridoxine, and 200mg/l calcium-D-pantothenate.

Filter-sterilised stress agent was added 44 hours after inoculation (unless otherwise stated) at the final concentration of: DTT 20mM and tunicamycin 10µg/ml. Control cultures had an equivalent volume of water added.

E. coli strains were grown in LB (Luria-Bertani) medium containing 1% (w/v) Bacto tryptone, 0.5% (w/v) Bacto yeast extract, 1% (w/v) NaCl, and 100 µg/ml Filter-sterilised. For LB agar plates 1.5% (w/v) agar was added.

2.4 Harvest of mycelia

Mycelia were harvested from 100 ml cultures by filtering cultures through two layers of sterilised Mira-cloth (CalBiochem, USA) and immediately flash frozen in liquid nitrogen. For dry weight determination the mycelia were freeze-dried in an Edwards Modulyo freeze drier for 48 hours before weighing, then mycelia were freeze-dried for a further 24 hours to ensure that they were completely dry.

2.5 Isolation and manipulation of DNA

Genomic *A. niger* DNA was isolated from 100 mg freeze-dried mycelia that had been ground under liquid nitrogen. The ground mycelia were resuspended in 3 ml of extraction buffer (200 mM Tris-HCl pH 8.5, 250mM mM NaCl, 25 mM EDTA), and 0.5% (w/v) SDS and extracted twice with phenol/chloroform after treatment with 5 mg/ml proteinase K at 65°C for 30 minutes. After centrifugation at 9700g for 15 minutes at 4°C DNA was precipitated by adding 0.7 volumes of isopropanol, 0.1 volume 3 M sodium acetate (pH 4.8) and centrifuged at 9700g for 30 minutes at 4°C. The pellet was washed with 70% (v/v) ethanol, air dried, and resuspended in 1 ml QB buffer (750 mM NaCl, 50 mM MOPS (3-(N-morpholino) propanesulfonic acid, pH 7.0) and the solution was purified through QIAGEN mini or midi columns according to the manufacturer's instructions (Qiagen UK Ltd). After the final centrifugation step the DNA pellet was air-dried, and then dissolved in 50 µl TE buffer (10 mM Tris, 1 mM EDTA, pH8.0). The presence of DNA in the extracted sample was checked by electrophoresis through 1% (w/v) agarose gels using 1X TAE (40 mM Tris, 1 mM EDTA, 2 mM sodium acetate (pH 7.5)) as running buffer. DNA solution were quantified spectrophotometrically from the absorbance at 260 nm ($OD_{260} 1=50\mu\text{g/ml}$ DNA) and stored at -20°C.

DNA manipulations using restriction and modification enzymes (Promega, UK), preparations of *E. coli* competent cells for heat shock and heat shock transformation were performed using standard procedures (Sambrook *et al.*, 1989). Purification of DNA fragments from restriction digests on agarose gels were carried out using a QIAquick kit (Qiagen, UK) according to the manufacturer's instructions.

PCR-generated fragments were ligated into the pGEM[®]-T Easy cloning vectors (Promega, UK). Plasmid DNA from *E. coli* was isolated by the Qiagen plasmid mini kit (Qiagen, UK) according to the manufacturer's instructions for subsequent identification of required clones.

2.6 Polymerase Chain Reaction (PCR)

50 ng of DNA was routinely used as template for PCR amplification in a 50 μ l reaction volume containing 0.2 mM dNTPs (final concentration each (Promega)), 20 pmol each primer, 10X PCR buffer, 25mM MgCl₂, and 2.5U Red Hot DNA Polymerase (ABgene, UK). PCR condition routinely consisted of 30 cycles with an initial denaturation step of 5 minutes at 94°C, 60 sec annealing of primers at a temperature usually 2°C below the T_m or as determined empirically using gradient PCR, and 90 sec at 70°C, followed by a final extension of 10 minutes at 72°C. The T_m was calculated as follows:

$$T_m = 2X(\text{sum of the primer A and T nt}) + 4X(\text{sum of the primer G and C nt})$$

Routinely, PCR products were purified using the QIAGEN PCR purification kit (Qiagen, UK) or agarose gel-extracted using the QIAGEN gel extraction kit (Qiagen, UK) following the manufacturer's instructions.

2.7 RNA extraction

Total RNA was extracted from 100mg freeze-dried, ground mycelia using the RNeasy Plant Mini Kit (Qiagen, UK) according to the manufacturer's instructions. DNA contamination was removed by treating RNA samples with RQ1 RNase-free DNase (Promega) according to the manufacturer's instructions and cleaned using an RNeasy column. Total RNA was quantified by reading absorbances at 260nm and 280nm. Ratios of over 1.9 for the 260nm:280nm readings were considered as an indicator of good quality RNA.

2.8 Northern blots and quantification of transcript level

For northern blotting, 5-7 μ g RNA per lane was run on a 7%(w/v) formaldehyde gel in 1X MOPS running buffer (0.2 M MOPS, 0.05 M sodium acetate, 0.01 M EDTA, pH 7.0). RNA samples were mixed with Sigma loading buffer (R4268) using a 2:1 ratio of buffer to sample. Samples were denatured at 65°C for 10 min then placed on ice for 5 min. RNA samples were electrophoresed against RNA markers (Promega) for either 16 hours at 25V or 4 hours at 65V. Following electrophoresis, RNA samples in the gel were visualised under UV light.

the formaldehyde gel was then washed 4 times in diethylpyrocarbonate (DEPC) (0.1% w/v) water for 10 minutes each. After the final wash, the gel was immersed in 0.05 M NaOH/1.5 M NaCl for 10 minutes then neutralised by soaking in 10X SSC (1.5 M NaCl, 150 mM sodium citrate) for 20 minutes. RNA samples were then transferred to Hybond N⁺ nylon membrane (Amersham Pharmacia Biotech) by a capillary system overnight using 10XSSC buffer. Next, the blot was washed once in 2XSSC for 2 minutes before UV cross-linking at 0.12 joules for 90 sec in a UV Stratalinker (Stratagene). The blot was left to air dry overnight, as a minimum, before probing. Procedures for northern blots were carried out in RNase-free conditions.

Blots were visualised using a Fujifilm BAS2000 phosphorimaging system. For quantification of transcripts, the amount of transcript from the gene of interest was calculated by dividing the value of the radioactive band (transcript) signal of that gene, after subtracting the background for individual bands, by that of the γ -actin probe which was used as an internal standard to normalise for loading variation. Change in the transcript levels of the target gene under specific treatment was determined by comparing the amount of transcript from the treated sample to untreated sample (control) for the same time point. When necessary, the transcript values were expressed as a percentage.

2.9 Probing and Hybridisation of the blots

Probes were amplified using *A. niger* genomic DNA and labelled using the Megaprime kit (Amersham, UK) and α -³²P-dCTP (Amersham, UK) according to the manufacturer's instructions. Primer sequences for amplifying all probes used in this study are shown in their relevant sections. The *glaA* probe was a 637bp long fragment corresponding to coordinates +1059 to +1696 of the *A. niger* glucoamylase gene (Boel *et al.*, 1984a). The γ -actin probe was a 765bp long fragment that corresponds to coordinates +889 to +1654 of the *A. nidulans* γ -actin gene (Fidel *et al.*, 1988). The *bipA* probe was a 445bp fragment corresponding to co-ordinates +712 to + 1156 of the *A. niger* *bipA* gene (van Gemeren *et al.*, 1997). For blot hybridisation, first the blot was prehybridised in 8 ml of hybridisation

solution (Puregene) at 65°C for 30 min. Next, the probe mixture (probe DNA, random primers, reaction buffers, dNTP, and α -³²P-dCTP) was added and hybridisation allowed to proceed overnight at 65°C. The blot was washed twice with 2XSSC containing 0.1% SDS for a total time of 20 min followed by one wash with 0.1XSSC containing 0.1% (w/v) SDS for 30 min. Both washes were carried out at 65°C.

2.10 β -Glucuronidase activity assay

Quantitative assays for β -Glucuronidase (GUS) activity from culture supernatants with 4-Methylumbelliferyl- β -D-glucuronide (MUG) were carried out as follows: mycelia were harvested as described in section 2.4 and approximately 500 mg fresh (wet) mycelia were ground under liquid nitrogen then resuspended in 1.5 ml extraction buffer (50 mM sodium phosphate buffer (pH 7.0), 1 mM EDTA, 5 mM β -mercaptoethanol, and 0.005% (w/v) Triton X-100). The mixture was centrifuged for 20 min at 10,500g and the clear supernatant was divided into two aliquots. One aliquot was used for GUS activity assays and the other for determination of the total protein concentration, after the addition of 1 mM phenylmethanesulfonyl fluoride (PMSF). All the steps were carried out on ice to minimize any proteolysis activities. Next, 50 μ l from one aliquot of the supernatant was mixed with 50 μ l of the reaction buffer (2 mM MUG and 100 mM sodium phosphate buffer (pH 7.0)) and immediately 10 μ l of this mixture was added to 190 μ l stop buffer (0.2 M Na₂CO₃), at zero time point. The remainder of the mixture was incubated at 37°C and 10 μ l aliquots were taken at 5, 10, and 15 minutes in the same manner.

Fluorescence was then measured with excitation at 365 nm, emission at 455 nm (Wallac 1420 VICTOR³™, PerkinElmer USA), and the resulting slope of the 4-methylumbelliferone (MU) fluorescence versus time was determined for each sample to calculate the GUS fluorescence per minute. The standard for the calibration was freshly prepared each time with the following concentrations of MU; 0.05, 0.1, 0.2, 0.4, 0.8, 0.16, 3.2, 6.4, 12.8 μ M in the same stop buffer and

used to calculate GUS activity in the assayed sample. The specific activity of GUS was expressed as GUS activity/mg protein, calculated after determining the total protein concentration (section 2.11). One unit of GUS activity is defined as the amount of the enzyme producing one μmol of methylumbelliferone (MU) per minute at 37°C.

2.11 Assay of total protein concentration

Total protein concentrations in fungal extracts were determined using the Bio-Rad Protein Assay kit, which is based on the dye-binding method of Bradford (Bio-Rad, USA), according to the manufacturer's instructions. Bovine serum albumin (BSA), diluted in the same extraction buffer used for the samples, was used as a protein standard for calibration. Assays were done in triplicate for each sample and averaged.

2.12 Nomenclature of strains

Genes from filamentous fungi are written in lower case italics, while the proteins are written in lower case, except the first letter, which is written in upper case and a letter or number usually follows this notation. Genes from mammals are written in upper case italics and proteins in upper case. For *S. cerevisiae*, genes are written in upper case italics while proteins are written in lower case except for the first letter, which is written in upper case and the convention followed by the letter p for proteins. Mutated genes in mammals and *S. cerevisiae* are written in lower case italic.

3 Activation of ER stress responses

3.1 Introduction

The highly oxidising environment of the ER promotes the formation of disulphide bonds, which are common in many secreted proteins. Disruption of the ER environment can hinder protein folding and results in accumulation of unfolded proteins in the ER. Accumulation of unfolded proteins is known to activate the UPR mechanism, which is likely to serve as a defence mechanism, to alleviate the harmful effects of the limitation in the capacity of the secretory pathway. Genome-wide analysis in yeast and mammalian cells using DNA micro-arrays has revealed that the UPR has broader effects than previously anticipated; however, this does not necessarily mean that all of these effects are direct. Genomic analysis studies under active UPR showed that, rather than regulating only ER-resident chaperones and foldases, as previously thought, the UPR affects the regulation of genes involved in nearly all stages of the secretory pathway (Benedetti *et al.*, 2000; Martinez and Chrispeels, 2003; Travers *et al.*, 2000). Furthermore, it has been demonstrated in yeast that UPR controls some physiological activities of the cell in response to nutrient availability. More specifically, in *S. cerevisiae*, the UPR is activated in the presence of high concentrations of extracellular nitrogen which then can repress two developmental programs of the cell, filamentous growth and sporulation, which are commonly activated as a response to nitrogen starvation (Schröder *et al.*, 2000).

There is a variety of environmental stress conditions that can disrupt protein folding and lead to accumulation of unfolded proteins in the ER. These conditions include: the use of chemical agents such as DTT, tunicamycin, or overexpressing of some homologous proteins or expressing heterologous proteins (Mulder *et al.*, 2004; Pakula *et al.*, 2003; Saloheimo *et al.*, 2003; Saloheimo *et al.*, 2004; Valkonen *et al.*, 2003a; Valkonen *et al.*, 2003b). High extracellular nitrogen concentration in yeast can activate the UPR possibly as a result of rapid protein translation which is likely to result in overloading of the secretion pathway.

Activation of the UPR also results in the activation of ERAD. The ERAD mechanism eliminates misfolded proteins via degradation in the cytosol, as has been reported in both yeast and mammalian systems and which was recently reviewed by Bonifacino and Weissman (1998). Gene expression analysis studies revealed an intimate coordination between ERAD and UPR: efficient ERAD requires an intact UPR, and UPR induction increases ERAD capacity. On the other hand, loss of ERAD leads to constitutive UPR induction (Travers *et al.*, 2000). The ERAD mechanism is mediated by the 26S proteasome which is composed of a multicatalytic 20S core particle (CP) (cylinder-like shape) and the 19S regulatory particle (RP) (cap-like shape) to the ends of the 20S core structure (Voges *et al.*, 1999). Recognition of ERAD substrates by the proteasome is mediated by a polyubiquitin-binding protein present in the 19S cap (Lam *et al.*, 2002) and then degradation occurs in the 20S core.

In this Chapter several environmental conditions that may result in activating ER stress responses were examined. These conditions include: treating *A. niger* cultures with chemical agents that interfere with protein folding (DTT, tunicamycin), using an *A. niger* strain with a functionally incomplete protein folding system (strain expressing antisense transcripts for the gene encoding PdiA), or by growing *A. niger* cultures in different nitrogen concentrations. Activation of ER stress was checked by means of *hacA* mRNA intron splicing and/or truncation, either by northern blotting or a more sensitive method, RT-PCR. Moreover, activation of the ERAD mechanism in DTT-treated *A. niger* AB4.1 cultures was demonstrated by detecting the transcription levels of the putative gene encoding the RpnG (MacKenzie *et al.*, submitted for publication, 2004), a homologue of the yeast Rpn7p subunit of the 26S proteasome.

3.2 Materials and methods

3.2.1 Primers used for preparation of DNA probes for northern analysis of transcripts levels

Table 3.1 shows the primer sequences used for amplifying all probes used in this Chapter. Also, the Table shows the PCR conditions used for the amplifications.

3.2.2 Isolating the mRNA and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted as described in Chapter 2 section 2.7. 60 µg DNase-treated (RQ1 RNase-Free DNase, Promega) total RNA was used to isolate mRNA using the Oligotex mRNA Mini Kit (Qiagen, UK) according to the manufacturer's instructions. First strand cDNA was synthesised using the Omniscript Reverse Transcriptase Kit (Qiagen, UK) with 1 µl (0.5 µg/µl) and Oligo(dT)₁₂₋₁₈ primers from the Universal Riboclone cDNA Synthesis Kit (Promega, UK) using 5µl isolated mRNA as template. PCR was carried out using 5 µl cDNA (from first-strand reaction) and the *hacA* primers located on either side of the 20 bp *hacA* intron (Table 3.1). PCR amplification was carried out in a final volume of 50 µl using Taq Red Hot DNA Polymerase (ABgene, UK) and PCR conditions as shown in Table 3.1. The 20 bp *hacA* intron splicing was detected by electrophoresing 7 µl of the PCR products through 3% (w/v) agarose gels with 100 ng ml/l ethidium bromide.

Table 3.1. Primers used for PCR amplification and the PCR conditions. All amplifications were carried out for 30 cycles with an initial denaturation step of 5 minutes at 94°C and a final extension of 10 minutes at 72°C.

Gene	Primers	PCR conditions	Experiment
actin	5'TCATGATCGGTATGGGTCAG3' 5'ACGATGTTGCCGTACAGATC3'	94°C / 30s 58°C / 60s 72°C / 90s	Northern
<i>bipA</i>	5'CAGAGACAGGCCACCAAGGACGC T3' 5'CGAACTTGGCGCGGGTGAGGGTC T3'	94°C / 30s 55°C / 60s 72°C / 90s	Northern
<i>glaA</i>	5'ATCTCTGGGAAGAAGTCAAT3' 5'CACAATAGAGACGAAGCCAT3'	94°C / 30s 58°C / 60s 72°C / 90s	Northern
<i>hacA</i>	5'TTGCCATTGTTGACAGT3' 5'CATAAGGAGAGACGTCA3'	94°C / 30s 48°C / 60s 72°C / 90s	Northern
<i>rpnG</i>	5'GATCCTTGCGGGCTCATTAT3' 5'CTAGCTCCGTTGCTTCCTC3'	94°C / 30s 53°C / 60s 72°C / 90s	Northern using poly ⁺ A- RNA
<i>hacA</i>	5'CTTCTCCTACCCTAACTCCT3' 5'TCAAAGAGAGAGAGGGCAC3'	94°C / 30s 53°C / 60s 72°C / 90s	RT-PCR

All probes were purified as described in Chapter 2 section 2.6.

3.2.3 Medium-exchange for improving tunicamycin treatment

A. niger cultures were grown as described in Chapter 2 section 2.1 in ACMS/N/P medium for 44 hours. After that, mycelia from 100 ml were filtered through two layers of sterilised Mira-cloth (CalBiochem, USA) and immediately transferred to a similar volume of new ACMS/N/P medium (pH 6.5) stored at the same temperature (28°C). Tunicamycin was then added (10µg/ml) to each flask, and the control cultures had an equivalent volume of water added. The

tunicamycin was prepared by dissolving in 10 ml TE buffer (pH 8.0) and 50 µl of 5M NaOH. Cultures were harvested as described in Chapter 2 section 2.4.

3.2.4 Isolation of poly⁺ A-RNA (including mRNA) for northern blots

Total RNA was extracted as described in Chapter 2 section 2.7. 150 µg DNase-treated (RQ1 RNase-Free DNase, Promega) total RNA was first divided into two aliquots, then each aliquot was cleaned by filtering through an RNeasy column (Qiagen, UK), according to the manufacturer's instructions. Two RNeasy columns were used for RNA cleaning because the maximum binding capacity for each column is 100 µg RNA. Next, cleaned total RNA was used to isolate poly⁺ A-RNA using the Oligotex mRNA Mini Kit (Qiagen, UK) according to the manufacturer's instructions and it was eluted in 20 µl RNase-free water. Extracted poly⁺ A-RNA samples were then used in northern blots as described in Chapter 2 section 2.8.

3.2.5 Media and growth conditions for studying the impact of low and high nitrogen concentrations in the growth media on *hacA* unconventional intron splicing

For detecting the impact of low and high nitrogen concentration in the growth media on UPR activation, *A. niger* AB4.1 cultures were grown in AMMN with starch as carbon source and ammonium chloride as nitrogen source. The AMMN medium contained 1% (w/v) starch, 20ml/l of ACM salts, 50 mM sodium phosphate buffer (pH 6.5), 0.375 % (w/v) NH₄Cl and 1% (w/v) uridine (after autoclaving). Cultures were grown for 38 hours, as described in Chapter 2 section 2.3. Next, cultures were filtered through Mira cloth (CalBiochem, USA) and washed in the same volume of AMM medium with no nitrogen source and transferred to AMM medium with either high (2.4 % (w/v)) or low (0.057 % (W/v)) NH₄Cl concentration. Samples were then collected at 0 (immediately after washing the mycelia), 15, 30, 60, 120, and 240 minutes after medium exchange, as described in Chapter 2 section 2.4.

3.3 Results

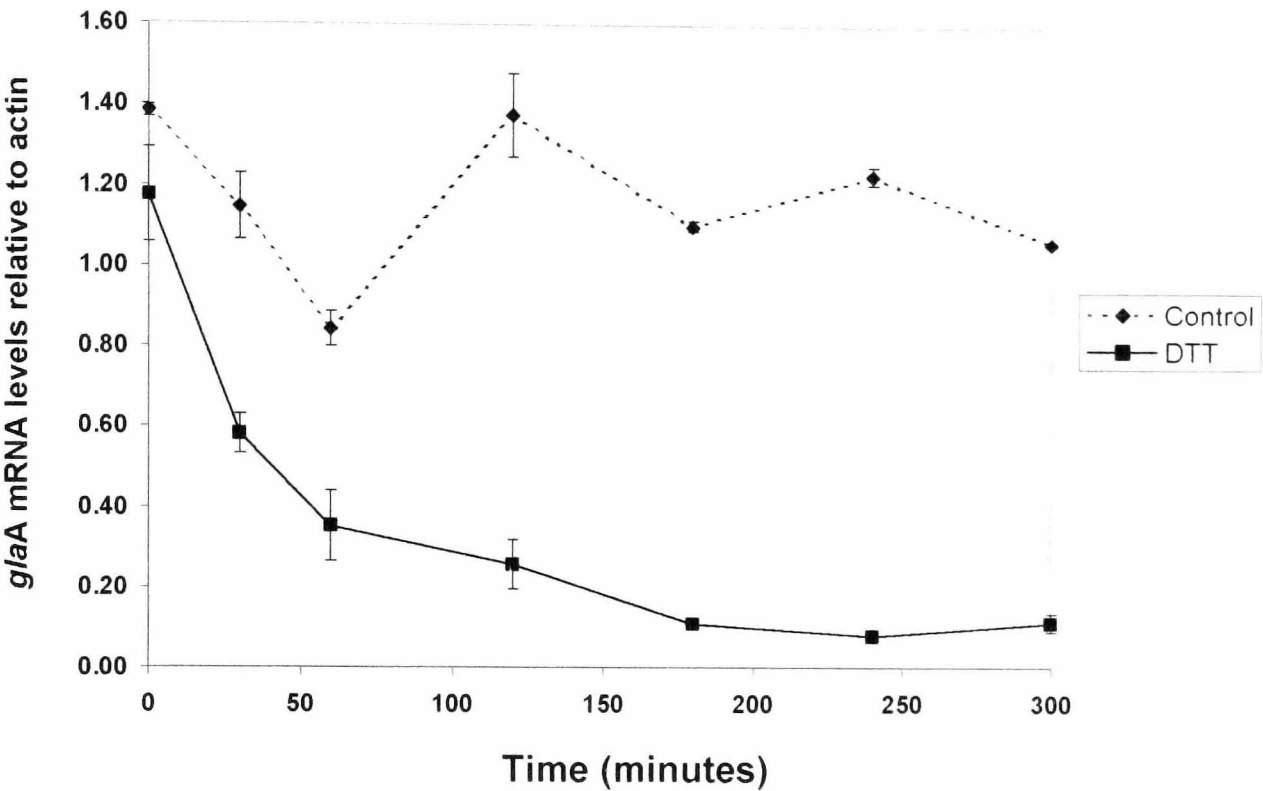
3.3.1 Decline in the transcript levels of the indigenous glucoamylase-encoding gene in DTT-treated cultures of *A. niger* AB4.1

Figure 3.1 (a and b) shows the effect of DTT treatment (20mM) on the transcript level of *glaA* mRNA over a time course of five hours. As shown in Figure 3.1, the level of *glaA* transcripts in DTT-treated cultures dropped over time. This result shows that, under stress conditions, caused by treating the cultures with DTT, the steady state level of *glaA* mRNA is lowered relative to that of actin mRNA. It is not known at this stage if the decline in *glaA* transcript levels is due to a transcriptional regulation or that DTT may increase the turnover rates of mRNA. However, the similarity between the declining rates of *glaA* mRNA when exposed to DTT (Figure 3.1) and that calculated in the absence of *de novo* synthesis of *glaA* mRNA as studied by medium exchange experiments (Al-Sheikh *et al.*, 2004) suggests that the decline of *glaA* mRNA levels is regulated at the transcriptional level, as the half-life of *A. niger glaA* mRNA under both conditions was approximately 80 minutes. It is known that exposure of filamentous fungi to DTT inhibits the formation of disulfide bonds which results in accumulation of unfolded protein and prevents their transport from this compartment (Ngiam *et al.*, 2000; Pakula *et al.*, 2003). Thus, DTT treatment results in ER stress by accumulating unfolded proteins in the ER and, thereby, activates the UPR mechanism. To monitor the UPR activation and to confirm that the ER is indeed under stress due to DTT treatment, the transcript levels of the gene encoding the molecular chaperone *bipA* were detected.

Figure 3.1

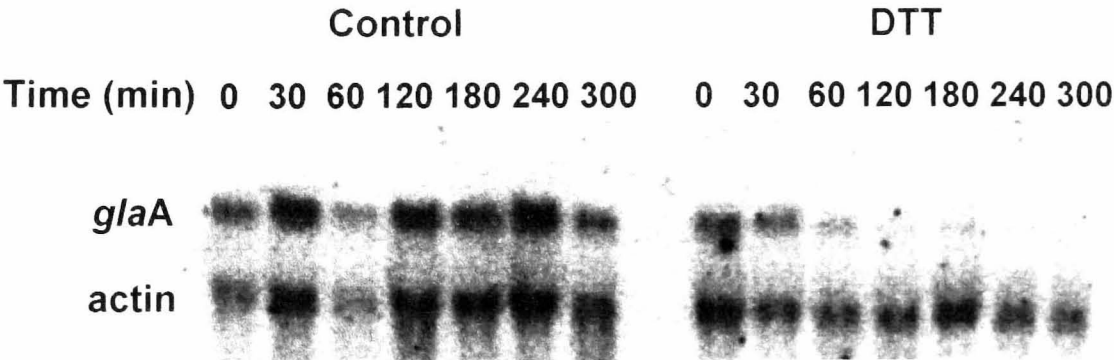
a.

The effect of DTT treatment on the transcript levels of *glaA*. Transcript levels of *glaA* mRNA were calculated as a ratio against the actin transcripts. The *glaA* expression levels are an average \pm SEM of three replicates.



b.

Northern blot of total RNA from DTT-treated cultures of *A. niger* AB4.1. The blot was hybridised with actin and *glaA* probes. Time periods shown refer to the time after DTT addition at which the samples were collected.



3.3.2 Activation of the Unfolded Protein Response mechanism in DTT-treated *A. niger* AB4.1 cultures

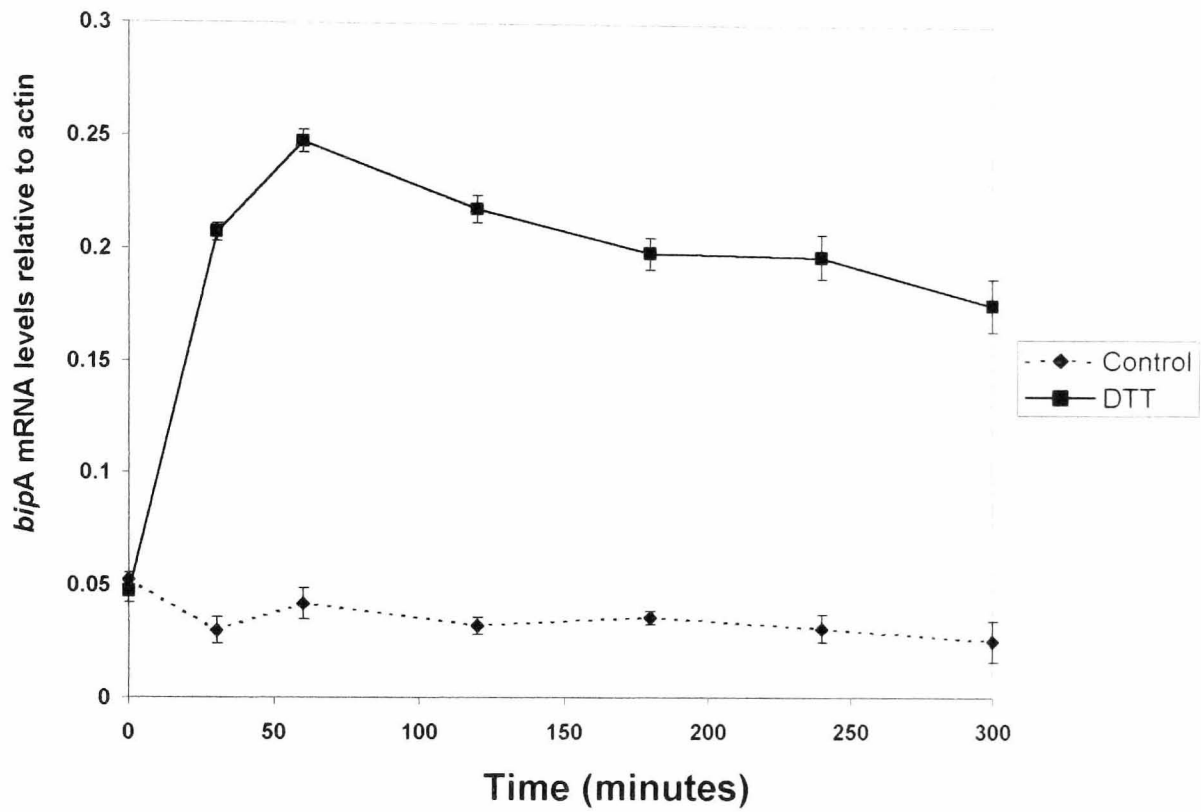
Figure 3.2 (a and b) shows the *bipA* transcript levels of *A. niger* AB4.1. In the *A. niger* DTT-treated cultures it is clear that the steady state *bipA* level is increased, indicating UPR activation. As shown in Figure 3.2 (a and b) *bipA* showed a rapid transcriptional response to the addition of DTT which induced *bipA* transcription by 6-7 fold in 30 minutes and remained high during the course of the DTT treatment. It is not known, however, whether the longevity of the raised level of *bipA* transcripts is due to the production of mRNA for an extended period caused by the persistence of the stress agent or because of a long half-life of the transcripts involved. In addition, the level of *bipA* transcripts was always checked in ER stress and medium-exchange experiments since the difference in the transcription level of *bipA* between stress and non-stressed samples is easily observed by looking at the band intensity in the gel.

To further confirm the UPR activation, the translationally active form of *hacA* mRNA was detected by northern blotting (Figure 3.3) and RT-PCR (Figure 3.7). Splicing of an unconventional intron and truncation of the mRNA at the 5' flanking region activate the synthesis of the HacA transcription factor, which acts as a positive transcriptional factor for genes regulated by UPR (Cox and Walter, 1996; Mori *et al.*, 1996). The latter event contributes mainly to the size difference seen in northern blots between the translationally active and non-active form of *hacA* mRNA in aspergilli. Figure 3.3 shows a northern blot of *hacA* mRNA. It can be seen that, in the DTT treated samples, splicing of *hacA* mRNA transcripts starts to take place in the first 30 min of DTT treatment. The same observation was not seen in the control samples, thus confirming the stress effect in the treated set of samples.

Figure 3.2

a.

The effect of DTT treatment on the transcript levels of *bipA*. Transcript levels of *bipA* mRNA were calculated as a ratio against the actin transcripts. The *bipA* expression levels are an average \pm SEM of three replicates.



b.

Northern blot of total RNA from DTT-treated cultures of *A. niger* AB4.1. The blot was hybridised with actin and *bipA* probes. Time periods shown refer to the time after DTT addition at which the samples were collected.

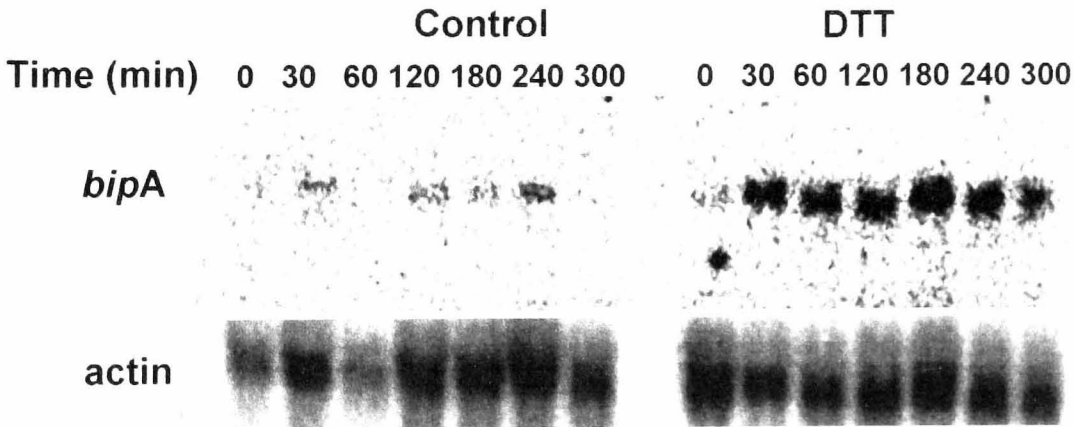
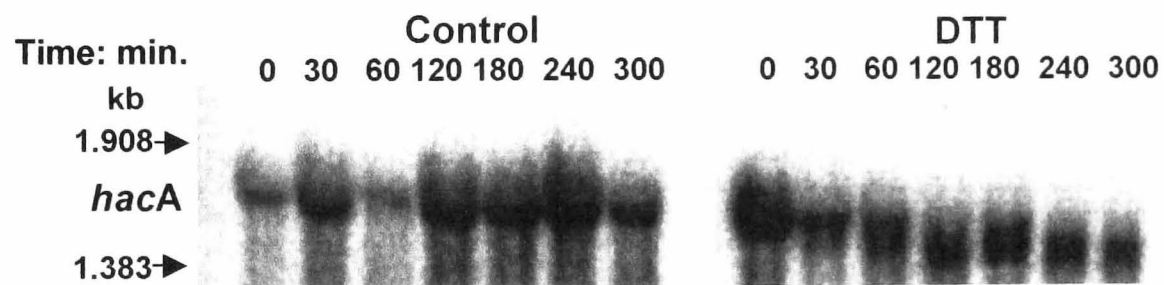


Figure 3.3

Northern blot of total RNA from DTT-treated cultures of *A. niger* AB4.1. The blot was hybridised with the *hacA* probe. Time periods shown refer to the time after DTT addition at which the samples were collected. The difference between the two bands is due to 5'-truncation of 230 bp (Mulder *et al.*, 2004).



3.3.3 Treatment of *A. niger* cultures with tunicamycin results in poor activation of the UPR compared to DTT

To further study the ER stress effect on the transcription of *glaA*, a different stress agent was used. Several experiments were carried out using tunicamycin as stress agent. First, Figure 3.4 shows that no *hacA* mRNA truncation could be detected by northern blot. Next, a more sensitive method for detecting *hacA* intron splicing (RT-PCR) was used as shown in Figure 3.5, but no clear difference in the splicing of *hacA* mRNA intron between the treated and the control samples could be detected. The observation that no *hacA* mRNA splicing could be detected in the tunicamycin treated samples using a very sensitive method for detecting the splicing product (RT-PCR) may suggest that the tunicamycin effect could be hindered by the acidity of the fungal culture at the time of adding the stress agents, which is 44 hours (Figure 3.6) (pH 3.7). To test whether the acidity of the fungal culture is affecting tunicamycin as a stress agent or not, a medium exchange experiment was carried out, as described in section 3.2.3 and *hacA* mRNA intron splicing was detected by RT-PCR. As can be seen in Figure 3.7, the intron of *hacA* mRNA was spliced in the tunicamycin treated sample, but it was not as strong as the one spliced by DTT (positive control) (i.e. the majority of *hacA* mRNA transcripts were not spliced), further confirming the poor effect of tunicamycin on UPR activation. Also, it can be seen that in the tunicamycin control sample (Figure 3.7) there was a low amount of *hacA* mRNA splicing, but clearly more than what has been seen in the control sample (negative control) with no medium exchange.

In addition, to cover the possibility that the tunicamycin may have a transitory effect (for a short time), *A. niger* AB4.1 strain was treated with tunicamycin, in a medium-exchange experiment, for a maximum of 2 hours during which samples were collected at 0, 10, 20, 30, 45, 60, 90, and 120 minutes. Total RNA was extracted from these samples and checked by northern blotting. As shown in Figure 3.8, no *hacA* mRNA splicing was detected at any time point in the

tunicamycin treated samples. These results, again, suggested that tunicamycin is a poor inducer of UPR in *A. niger*.

Figure 3.4

Northern blot of total RNA from tunicamycin-treated cultures of *A. niger* AB4.1 strain. The blot was hybridised with the *hacA* probe, comprising the region from the start codon up to the non-conventional intron. Time periods shown refer to the time after tunicamycin addition at which the samples were collected. No *hacA* truncation could be detected as shown by northern.

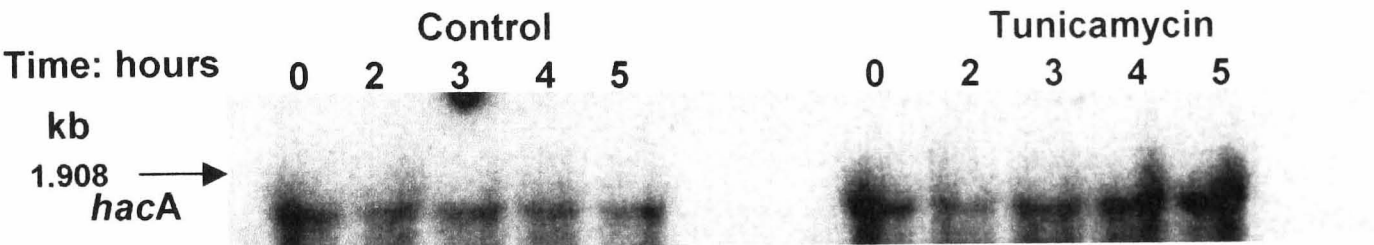


Figure 3.5

RT-PCR for *hacA* mRNA *A. niger* treated with tunicamycin (Tm). Strains were grown in starch medium for 44 hours. No medium-exchange was carried out before adding the Tm for 5 hours.

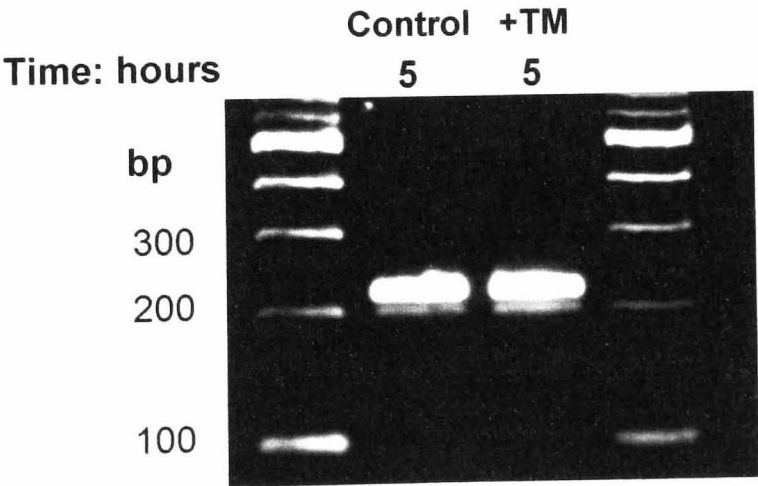


Figure 3.6

Dry weight and pH for *A. niger* AB4.1 in ACMS/N/P, ACMG/N/P or ACMX/N/P. Cultures were grown for 96 hours. Standard errors were generated from triplicates.

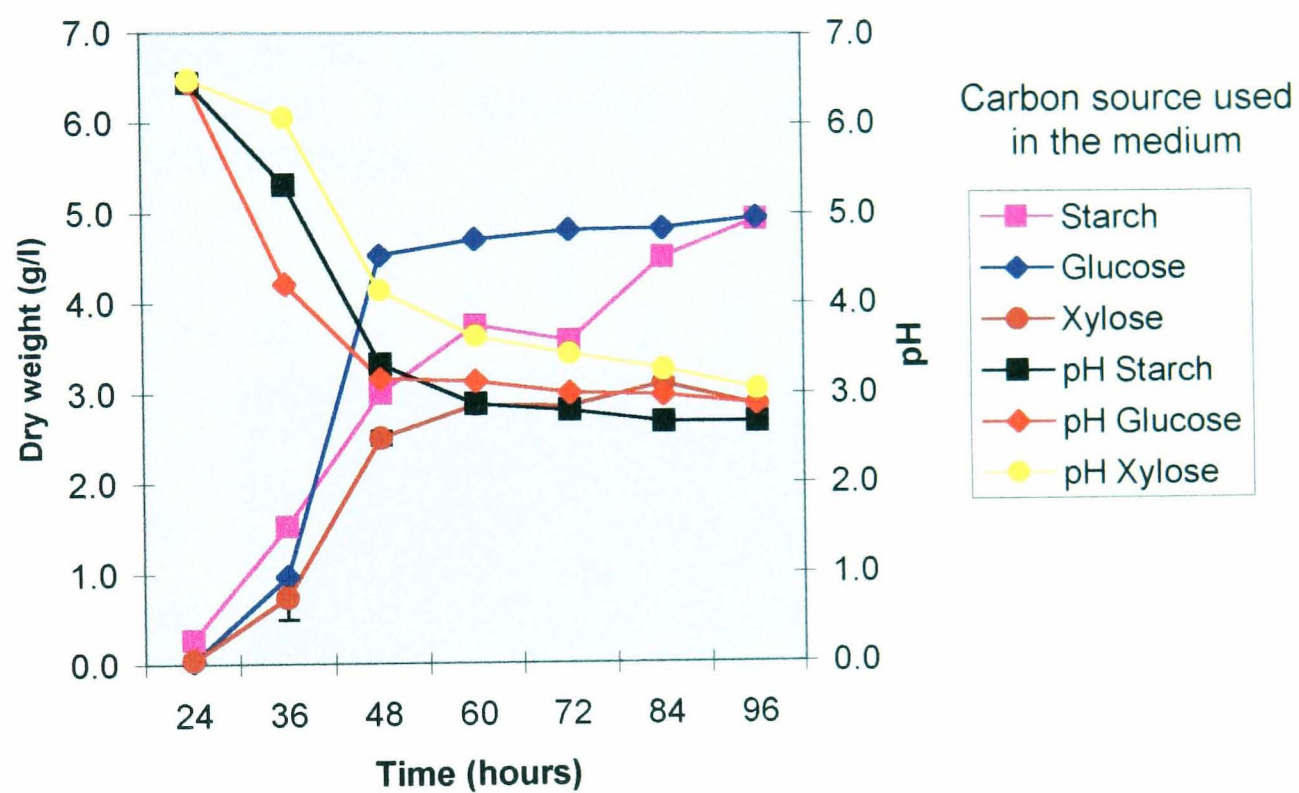


Figure 3.7

RT-PCR analysis of the *hacA* transcript across the 20 bp intron of *A. niger*. For control sample, no medium exchange was carried out. Samples were collected after 6 hours treatment of DTT. For tunicamycin-treated samples, tunicamycin was added after medium exchange, and then samples were collected after 5 hours of tunicamycin treatment. For the *pdiA* antisense strain AS1.1 and HEWL-producing strain B1, samples were collected at 48 hrs. Abbreviations: M: DNA marker, Cont.: control, Tm: tunicamycin. Tm cont.: control for medium-exchange.

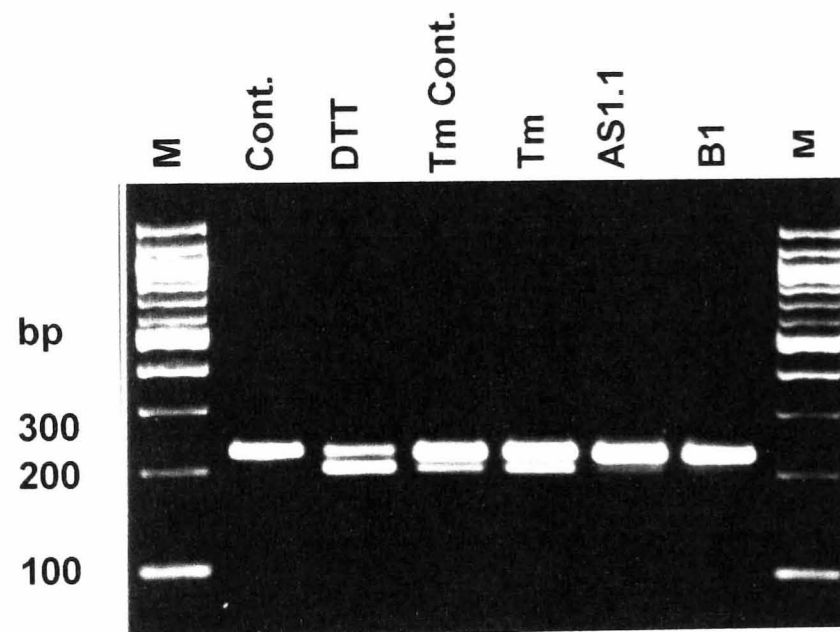
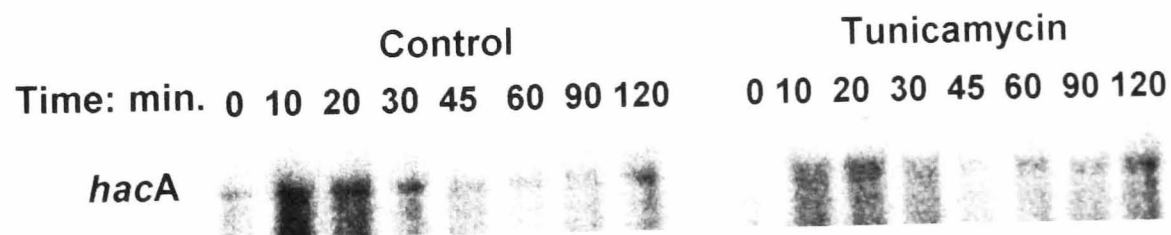


Figure 3.8

Northern blot of total RNA from tunicamycin-treated cultures of *A. niger* AB4.1. The blot was hybridised with the *hacA* probe. Time periods shown refer to the time after tunicamycin addition at which the samples were collected. No *hacA* mRNA truncation could be detected as shown by northern.



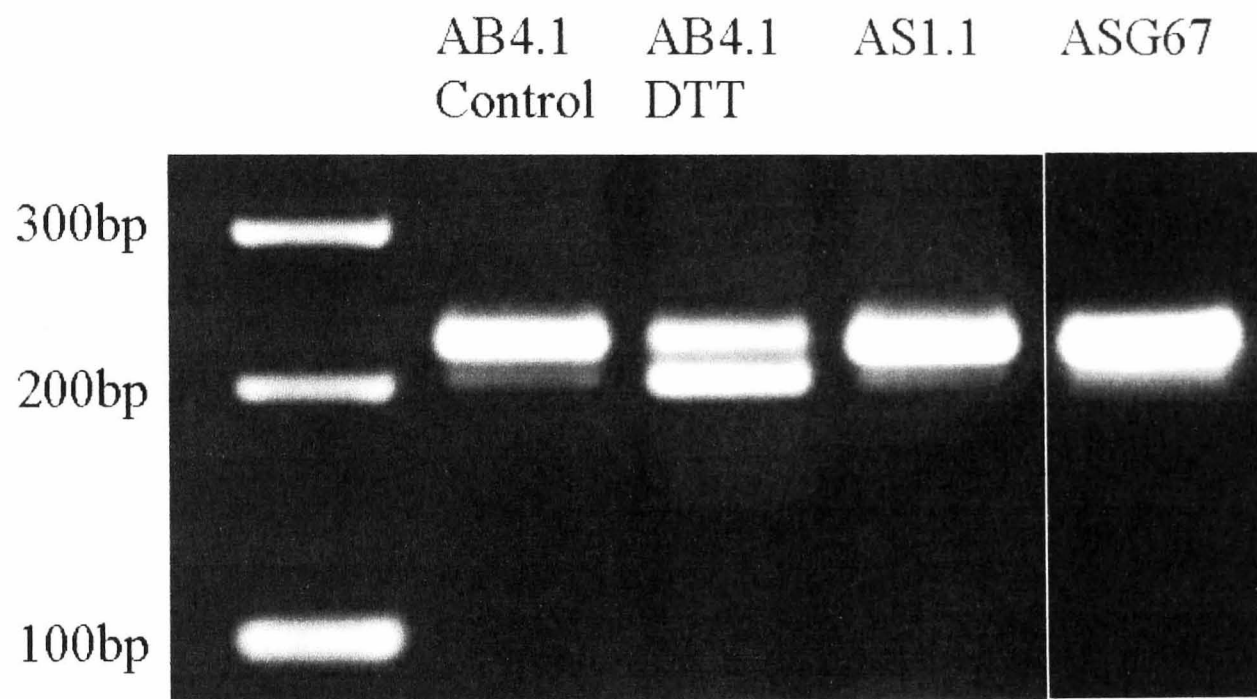
3.3.4 No obvious splicing of the unconventional *hacA* mRNA intron in *A. niger* strains AS1.1, ASG67 both with reduced level of PdiA, and *A. niger* B1 producing a heterologous protein

The *A. niger* AS1.1 is a multi-copy transformant containing 4 to 6 copies of *pdiA* antisense cDNA under the control of the *glaA* promoter and the *Aspergillus nidulans trpC* terminator (Ngiam *et al.*, 2000). The *A. niger* ASG67 also contains the *pdiA* antisense cDNA, but under the control of the constitutive *gpdA* promoter and the *Aspergillus nidulans trpC* terminator (Al-Sheikh *et al.*, 2004). It has been previously shown that the antisense *pdiA* strains exhibit lowered levels of PdiA foldase activity and lowered levels of secreted glucoamylase (GAM) (Al-Sheikh *et al.*, 2004; Ngiam *et al.*, 2000). It has been demonstrated previously that *glaA* transcript levels in the antisense *pdiA* strain AS1.1 decline over a growth period of 72 hours. To test whether this decline in *glaA* transcript levels is due to the activation of the UPR, *hacA* mRNA splicing was tested by RT-PCR.

Figure 3.9 shows the RT-PCR for detecting *hacA* mRNA splicing in AS1.1, ASG67, and AB4.1. As shown in Figure 3.9, no difference could be seen between the control and the *pdiA* antisense strains AS1.1 and ASG67. The observation that a low level of *hacA* mRNA splicing is present in these strains, including the parent strain (control), may indicate that the UPR is constitutively active at a very low level, probably to maintain the normal level of chaperones and foldases in the ER compartment. In contrast to the AS1.1 strain, a strong *hacA* mRNA spliced product can be seen in *A. niger* when exposed to DTT. This observation that the UPR is active only in the DTT-treated strain (Figure 3.7, and Figure 3.9), but not in the AS1.1 strain as judged by northern blotting and RT-PCR suggests that the decline in *glaA* transcripts is not mediated by UPR. Furthermore, the *A. niger* B1 strain, which overexpresses HEWL protein in ACMS/N/P medium, was checked by RT-PCR for *hacA* mRNA splicing to determine whether heterologous protein expression from this strain would result in UPR activation. As shown in Figure 3.7 *hacA* mRNA splicing is similar in this strain to that of its parent strain AB4.1

Figure 3.9

RT-PCR analysis of the *hacA* transcript across the 20 bp intron of *A. niger* AB4.1 (parental strain) treated with water (control) or DTT, *A. niger* AS1.1 and ASG67 (antisense *pdiA* strains). The AB4.1 DTT samples were grown as above but treated with 20 mM DTT for 6 h before harvest. The AS1.1 and ASG67 cultures were grown for 48 h and then harvested.



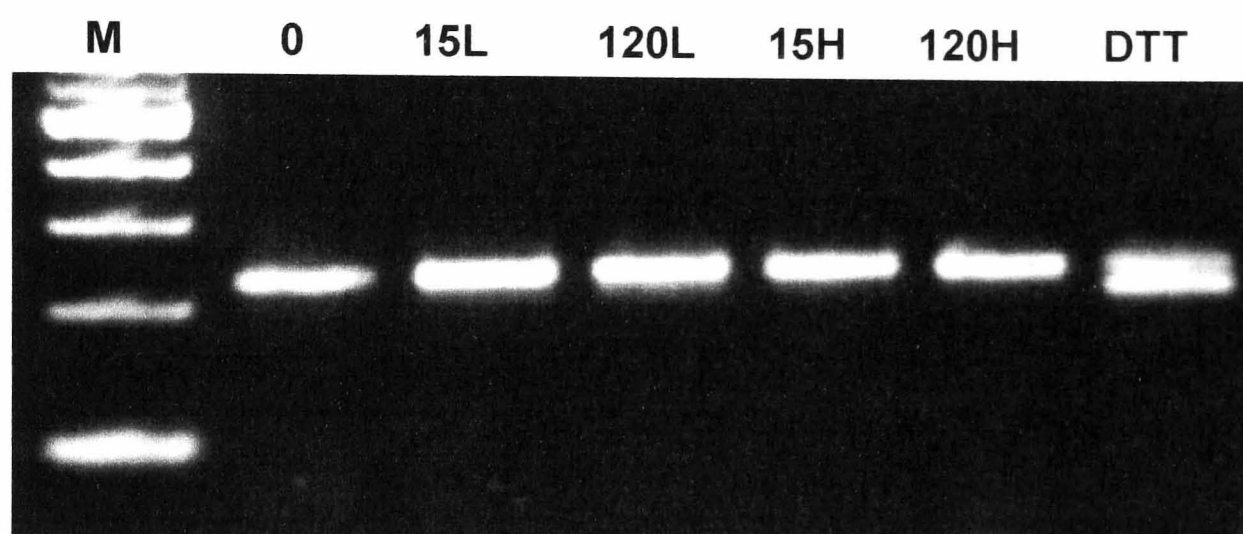
(control sample) and thus the UPR was not activated at least to a degree similar to that triggered by DTT.

3.3.5 No obvious splicing of the unconventional *hacA* mRNA intron in *A. niger* AB4.1 cultures grown in medium with low and high concentration of nitrogen

It has been observed in *S. cerevisiae* that diploid cells exhibit two developmental changes in response to nitrogen starvation. First, nitrogen starvation in the presence of a fermentable carbon source, such as glucose, results in a morphological change from yeast to filamentous growth form. Second, nitrogen starvation in the presence of nonfermentable carbon sources, such as acetate or ethanol, induces sporulation. However, it has been observed that both responses are repressed by activation of UPR (Schröder *et al.*, 2000). It was demonstrated in the above study that *HAC1* mRNA splicing is brought about by extracellular nitrogen levels (1 mM ammonium sulfate), which then can activate the UPR. This effect ceases rapidly, as early as 5 minutes, upon nitrogen starvation (Schröder *et al.*, 2000). It was proposed that in nitrogen-rich media, protein unfolding is a byproduct of rapid translation, resulting in low-level activation of the UPR and subsequent repression of filamentous growth and sporulation. When the nitrogen level drops, the amount of unfolded protein in the ER is no longer sufficient to activate the UPR and the repression of development changes is relieved. To test whether nitrogen concentration in the extracellular medium of *A. niger* can activate the UPR, *hacA* mRNA splicing was checked by RT-PCR in the presence of high (2.4 % (w/v)) or low (0.057 % (w/v)) NH_4Cl concentration as described in section 3.2.5. As Figure 3.10 shows, no *hacA* mRNA splicing could be detected after changing the concentration of NH_4Cl for either 15 or 120 minutes. This may suggest that the secretion pathway in *A. niger*, which is believed to have higher secretion capacity compared to that of *S. cerevisiae*, can tolerate activation of UPR due to these changes in the nitrogen concentration at least during the time points used.

Figure 3.10

RT-PCR analysis of the *hacA* transcript across the 20 bp intron of *A. niger*. Sample of 0 time-point was collected prior to the medium-exchange. Numbers refer to time in minutes after the medium-exchange before harvesting. L and H refer to low and high NH_4Cl , respectively.



3.3.6 Activation of the ER-Associated Degradation (ERAD) mechanism in DTT-treated *A. niger* AB4.1 cultures

A subtractive PCR library was made to contain differentially transcribed mRNAs from *A. niger* challenged with DTT (Watson *et al.*, 2000). DNA sequence analysis for some of these clones showed them to have sequence similarity to yeast genes involving the ERAD pathway (MacKenzie, personal communication). BLAST DNA sequence analysis (using the NCBI web site: <http://www.ncbi.nlm.nih.gov>) of some of these clones revealed sequence similarity to the 26S *RPN7* gene from the yeast *S. cerevisiae* involved in the ERAD pathway (MacKenzie, personal communication). A full length of the putative *rpnG* gene is shown in Figure 3.11. The nucleotide sequence data are present in the EMBL database under the accession number ANI575130.

To further study the transcriptional regulation of the gene encoding the putative 26S proteasomal regulatory subunit *rpnG* under ER stress, northern blots were performed. Attempts to use the total RNA for northern blotting failed to clearly detect the putative *rpnG* signals due to non-specific hybridisation with RNA ribosomal bands. For this reason, poly⁺ A-RNA (isolated as described in section 3.2.4.) was used instead. Figure 3.12 shows that transcript levels of putative *rpnG* were induced by 3-4 fold relative to actin transcripts after exposure to 20 mM DTT. However, due to the fact that the ORF of the two probed genes (*rpnG* and actin) are close in size, partial overlapping of these two bands resulted (Figure 3.12b). This problem was solved by first probing with *rpnG* as shown in Figure 3.13b (top panel). Next the blot was probed with actin (Figure 3.12b second panel). In order to calculate the signal intensity of the actin band, the whole large band, which contains actin and what had not decayed from the previously probed band of *rpnG*, was quantified. Thus, dividing the value of the *rpnG* band (probed first) by the whole band (actin + decayed *rpnG* band) would still keep the final calculated band of *rpnG* in ratio to actin.

Transcripts were calculated as a ratio:

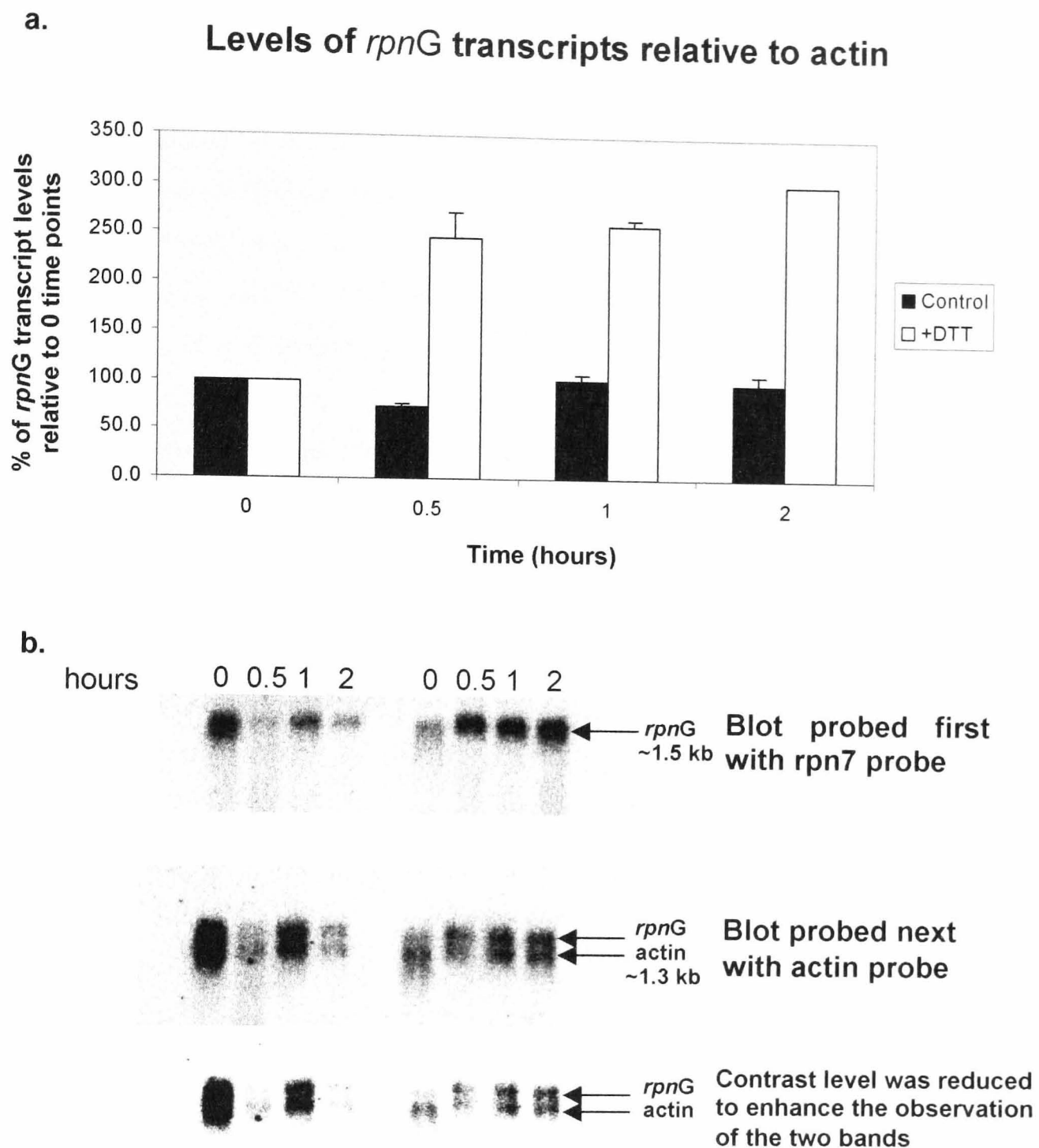
$$\frac{(\textit{rpnG absolute value})}{(\text{actin} + \text{decayed } \textit{rpnG})}$$

The induction of the putative *rpnG* transcripts under DTT treatment suggest that ERAD in *A. niger* is activated under ER stress conditions to degrade unfolded or misfolded proteins in a way similar to that observed in yeast (Travers *et al.*, 2000).

Figure 3.11 Sequence of *A. niger rpnG* gene and flanking regions. The nucleotide sequence data are in EMBL database under the accession number ANI575130 (MacKenzie *et al.*, submitted for publication, 2004). Introns and non-translated sequences are underlined; the translated sequence is given in lower case (not underlined); * (over) indicates the polyA signal and ^ (over) indicates the polyA site.

<u>ggctttccgc cacatcttca atgatctggt ggaaactgca cccttaactg accttatatc</u>	60
<u>gaattaatac catgcaggca ttaaagctct gattcatgcg aaactggcaa tccagctgga</u>	120
<u>aggcagcagc atcaatgaaa ggggtattaa gttgcgatcc acggttttag ctgccagtga</u>	180
<u>tccagttgcg tttggttatt agcctggccg gtgagcgcaa gttgtgggtc cttcagtcca</u>	240
<u>acccaaaaat tagattcatt ttacttccag ttcttactaa cggaattcc aacaaaaaca</u>	300
<u>gtcactcccg tggatgaatc ctctggtacg tagtatacgc gcaacaccct cgcggtgaca</u>	360
<u>ggaagattgt aaaggatcgg acattcgatg atggtttaca tccaatagaa tgcatacata</u>	420
<u>atacgtctac tactgtatcc tccattgctc ttccggtgtc tggccataag cacagtcatt</u>	480
<u>aacactgccc tatacggttg cagaaataat gaaaaggacg ataacttttc ggcatcagta</u>	540
<u>catgtaggac gttgtataat ggtgcatgtc agacgtaata attaaaggcg tgagaagcgt</u>	600
<u>tcgctggcgt tgtgttcgac ggcttgcctc catgaacgat ataatacgtg tagcatccct</u>	660
<u>agtgccaca aacattaact aaacgttcag agagccatcg ttctctagaa atgagtcttt</u>	720
<u>caccagata agctcgactt tgtttacgtc tcgatgggtg tcagcctata aactgttcga</u>	780
<u>atggctgttg aaacctggaa gcggttcgag cagccggaac aacatttccc cagctgagct</u>	840
<u>cgggtcagct acgttccata ggtgatgtat ctgcctcacc catcatcaac cccagaccct</u>	900
<u>ctgctcactt gacctcaag cttcgctcca tagcctcttc atttcaactt gcctatacgc</u>	960
<u>atctctttca tagctcgata tctaccccc atttgtcaac atggggtcgg atccgcagta</u>	1020
<u>cataaagtac cctgatctca acctcgcca acatgtcttc aacctctcca accctgcatg</u>	1080
<u>cccgcaaca gtgcgacaga catcattgaa gagacttcaa gatgttatct ccgagaacaa</u>	1140
<u>gatggctcca ttctaccgac acctcgctca tcccggtgaa ggcattctga accactctgg</u>	1200
<u>tgaagggtgc gccagcacc cgaccaagtc cctgattacg tcaaatatgc tggcttcgcg</u>	1260
<u>gaagtcgctc cagaacatag actttccctg ggatgaatcc ctgtaccagt cattggtgga</u>	1320
<u>agacaaccgg aaggagttgg aagccttcca aaaggaggag gatgaagctg aggaagccgc</u>	1380
<u>cggggaaaact gaggtgctgg ccgcacgggg taagcgcgcc gagttctggg cacgtgtagg</u>	1440
<u>cgataaggta tgtcttgaga agcttcagct aggagtctat ccgagataag ctctgcatga</u>	1500
<u>tgggtgattt gctaacctca gttcgtggct ggcaggacaa agctatcgaa tcccacgagg</u>	1560
<u>ccctccttga gaagacgaca ttctcggaa ccaagatcga cctgatgctg gctataatcc</u>	1620
<u>ggattggcct tttcttcggg gataccatt ctgtcaagaa gagcattgag cgggcaaca</u>	1680
<u>ctctcattga aagtggagga gactgggacc ggagaaaccg cctcaaggca tacaagggcc</u>	1740
<u>tacatctact caccatccgt tcatacagtc ttgctgcccc tctgctcctg gacagtctat</u>	1800
<u>cgaccttcac cagctacgaa ctgtgcagct actcgccctt ggtcatctat tcgatccttg</u>	1860
<u>cgggctcatt atccctgaag cgagtcgact tcaaggcaaa ggttgtggat gcacccgaga</u>	1920
<u>tcaaggccat tcttggaact ggagaggacc gcctagctgc tctgaccggc gagatatact</u>	1980
<u>ccgggcccgg tgcccaggat gaggagatga aggatgctac tgtcaccaga cgggcgccga</u>	2040
<u>ccacggctac cactgtggtt aacctacta ctctgggagc gggatctggc atccagtctg</u>	2100
<u>aagccgaagc gcccggtggac ttttcgcctc ttgcaaacct ggtagacagc ctgtacaccg</u>	2160
<u>gcaactaccg gaatttcttc gtagcgctgg cagccgtgga ggacaacttc ctgaaccagg</u>	2220
<u>accgttactt gcacgaacac cgcgcgtggt ttgtgcgcga gatgagactt cgtgcgtacc</u>	2280
<u>agcaactgct gcagagctat cgcgttggtg gcctgaacag catggccagc gactttggcg</u>	2340
<u>tcactgtaga ctacctggat cggtaagtcc gccattggac cagtatcggg tagaactgtg</u>	2400
<u>ttgactgaca gctacaggga tcttgccaaa ttcatgtcca gcaaccggat tgcattgcacc</u>	2460
<u>atcgatcggg tcaatggcat catcgagacg aaccgaccag acgacaagaa caagcagtac</u>	2520
<u>gcggatgttg tgaagcacgg cgatgccctg attacgaaac tgcaaaagta cggacaagcc</u>	2580
<u>gtgcggttgc gaggaagcga acggagctag aggggggttct gccagggacg gagtggctctg</u>	2640
<u>atcttgactc accgcacatg gtttatgact cgctagatga gagactcagt gatttatata</u>	2700
<u>tcttttgaca ccgttgacca ttgacactgc gagcgtccag acacgtgagg ctgctgtatc</u>	2760
<u>atacatataa tgacctgaag acaacatgac</u>	2790

Figure 3.12



The effect of DTT treatment on the transcript levels of a putative gene-encoding the subunit of the regulatory particle of the proteasome (*rpnG*). poly⁺ A RNA was probed with the putative *rpnG* probe and loading was normalised by probing with actin.

3.4 Discussion

The level of *glaA* transcripts in DTT-treated cultures of *A. niger* dropped over the time period examined. The similarity between the declining rates of *glaA* mRNA when exposed to DTT and that calculated in the absence of *de novo* synthesis of *glaA* mRNA as studied by medium exchange experiments (Al-Sheikh *et al.*, 2004) suggests that the decline of *glaA* mRNA is regulated at the transcriptional level, as the half-life of *A. niger glaA* mRNA under both conditions was similar. It is not known at this stage, however, if the decline in the *glaA* transcript level is operating at the transcriptional level or that DTT may increase the turnover rates of *glaA* mRNA. This is further addressed in the following chapters. When studying ER stress, many studies have used more than one stress factor to gain information more specific to UPR. For instance, studies performed with yeast, Arabidopsis, and human systems (Travers *et al.*, 2000; Martinez and Chrispeels, 2003; Benedetti *et al.*, 2000), respectively, have used both DTT and tunicamycin to treat cells under study to gain information more specifically related to UPR, by eliminating genes that are affected by only one stress agent. In addition, expression of a heterologous protein is also used in filamentous fungi to gain information more specific to UPR (Mulder *et al.*, 2004; Pakula *et al.*, 2001). However, the expression of heterologous proteins does not induce a clear UPR stress all the time. For example, the UPR mechanism is activated in both *A. niger* and *T. reesei* strains producing the heterologous protein t-PA (Mulder *et al.*, 2004), (Saloheimo, personal communication). On the other hand, the *bipA* level, which is induced when UPR is activated, remained unchanged when the heterologous gene encoding interleukin-6 was expressed in *A. niger* (Punt *et al.*, 1998).

Results generated from experiments presented in this Chapter showed that DTT is a strong stress agent that can activate the UPR as checked by *hacA* mRNA truncation and intron splicing and induction of *bipA* transcripts, whereas tunicamycin is a poor stress agent in *A. niger*. Moreover, it has been recognised in some recent studies that tunicamycin is a poor inducer of UPR in fungal strains

(Pakula *et al.*, 2003). The observation that no *hacA* splicing could be detected in the tunicamycin-treated samples using a very sensitive method for detecting *hacA* mRNA intron splicing (RT-PCR) may suggest some possibilities. Firstly, it is possible that no effect of tunicamycin has taken place due to unknown differences in carrying out the experiments in this study, and where tunicamycin is reported to induce UPR (Mulder *et al.*, 2004). Tunicamycin has been used as an ER stress agent in *A. niger* (Mulder *et al.*, 2004) with a medium-exchange prior to the addition of the tunicamycin to the samples. But tunicamycin showed variable results in that group's studies (Mulder, personal communication), and did not cause splicing of *hacA* mRNA each time. Although exchanging the media may slightly stress the fungus, as described below, it was avoided here in the first instance because changing the medium is likely to result in further shocking the cells at least for the next few hours after the medium-exchange. A second possibility is that the tunicamycin effect could be hindered by the acidity of the fungal culture at the time of adding the stress agents. Finally, the fungal cells may cope with the stress caused by tunicamycin without fully activating the UPR. To test whether the acidity of the fungal culture was affecting tunicamycin as a stress agent, a medium exchange was carried out as described in section 3.2.3. Tunicamycin treatment after exchanging to a new medium with higher pH (6.5) led to detection of some *hacA* mRNA splicing, but it was not as strong as the one caused by DTT, further confirming the poor effect of tunicamycin on UPR activation. Also, it can be seen that in the medium-exchange control sample (Figure 3.7), there was a low amount of *hacA* splicing, but clearly more than what has been seen in the control sample with no medium exchange. In addition, since the spliced and unspliced *hacA* mRNA are detected in the same mRNA sample, the amplification rate of these products will depend on their initial quantity in the RNA sample and if a difference could be seen in the intensity of these bands when checked in the agarose gel, it means that at least one of the band has not reached the saturation level of PCR amplification. Thus, determining the region of RT-PCR linearity cycles might not be critical if not comparing the RT-PCR products from different RNA samples.

The *A. niger* transformant strain AS1.1, with a functionally incomplete protein folding system, may have ER stress as a result of the presence of 4 to 6 copies of a *pdiA* antisense sequence under the control of the *glaA* promoter. Although the kinetics of growth and final yield of biomass were not significantly different in this antisense *pdiA* strain compared to its parental strain AB4.1 when grown in ACMS/N/P medium, the *glaA* transcript level in the antisense *pdiA* strain AS1.1 declined over a growth period of 72 hours (Al-Sheikh *et al.*, 2004). By using a sensitive transcript detection method, RT-PCR, it was clear that the UPR is not likely to be responsible for mediating the decline of *glaA* transcript levels. This observation suggests the existence of a UPR-independent pathway that mediates the decline of *glaA* transcripts. Further support for this suggestion comes from a recent study by Pakula *et al.* (2003). In this study it was found that treatment of a *T. reesei* culture with the ionophore A23187 resulted in a slight effect on UPR but significantly reduced the *cbh1* mRNA level. Moreover, in the above study a protein synthesis inhibitor cycloheximide (CHX) was used to check whether a reduction in protein synthesis, and probably in the ER protein load, would affect the UPR induction and the decline of transcripts of genes encoding secreted proteins in *T. reesei* (Pakula *et al.*, 2003). Results showed that DTT treatment in combination with the CHX resulted in no activation of the UPR as confirmed by the observation that no *hac1* mRNA truncation was detected nor was there induction of the UPR target gene *pdi1*. However, the level of the *cbh1* declined slightly in both treatments (DTT in combination with CHX or in cultures treated with CHX solely), whereas no UPR activation took place. This observation that the *cbh1* transcript level declined in the absence of UPR may suggest that the decline in the *cbh1* transcript level was mediated by a UPR-independent pathway.

High nitrogen concentrations in the extracellular medium have been observed to activate the UPR in *S. cerevisiae* (Schröder *et al.*, 2000). It was proposed that a high level of nitrogen in the extracellular medium results in rapid protein translation which then may overload the secretion pathway and activate the UPR. However, varying the concentration of nitrogen in the extracellular media of *A. niger* cultures did not result in UPR activation. *A. niger* may have a

higher secretion capacity compared to that of *S. cerevisiae* which could allow it to tolerate activation of UPR due to a change in the nitrogen concentration at least during the time course used in this study. However, it might be possible that the nitrogen concentration in the AMMN (0.375 % (w/v) NH_4Cl) which was used initially to grow *A. niger* strains is already high. Thus, it might be interesting to repeat this experiment by pre-growing the *A. niger* strains at lower nitrogen concentrations before transferring them to a media with low or high nitrogen concentrations. Also it might be important to do this experiment with different carbon sources. Schröder *et al.* (2000) reported that in *S. cerevisiae*, on a complex carbon source, the *hac1* mRNA splicing was increased and it was suggested that this might occur as a result of less efficient synthesis of ATP or related core oligosaccharide synthesis under these conditions. More specifically, on a glucose about 1%-3% of *hac1* mRNA was spliced whereas the amount of spliced *hac1* mRNA was increased 5- to 10-fold during growth on ethanol or acetate. Moreover, growth on a glucose-repressible carbon source such as maltose or raffinose resulted in a twofold increase in *hac1* mRNA splicing (Schröder *et al.*, 2000). Thus, it might be important when carrying out this experiment to study the impact of nitrogen concentration on the activation of the UPR in *A. niger* to use both simple and complex carbon sources.

In yeast, genome-wide analysis showed an intimate coordination between ERAD and UPR, where efficient ERAD requires an intact UPR, and UPR induction increases ERAD capacity. Up to the present time, the only 26S proteasomal subunit gene identified from *A. niger* has been that which encodes the homologue of the yeast regulatory particle Rpt3p subunit (TbpA), which is a member of the AAA-ATPase family (Jarai, G., unpublished data; Swiss-Prot P78578, MacKenzie, personal communication). A partial DNA (468 bp) sequence of a gene that was obtained from a subtractive PCR library challenged by DTT in *A. niger* (Watson *et al.*, 2000) showed a high homology to the sequence of a gene encoding the 26S proteasomal regulatory subunit *rpn7* of *S. cerevisiae*. Northern blots showed that the transcript level of the putative gene encoding the 26S proteasomal regulatory subunit *rpnG* in *A. niger* increased 3-4 fold after 30 minutes of exposure to DTT,

which suggests that ERAD in *A. niger* is possibly activated under ER stress conditions to degrade unfolded or misfolded proteins in a way similar to that observed in yeast (Travers *et al.*, 2000).

4 Role of the glucoamylase promoter (P_{glaA}) under ER stress

4.1 Introduction

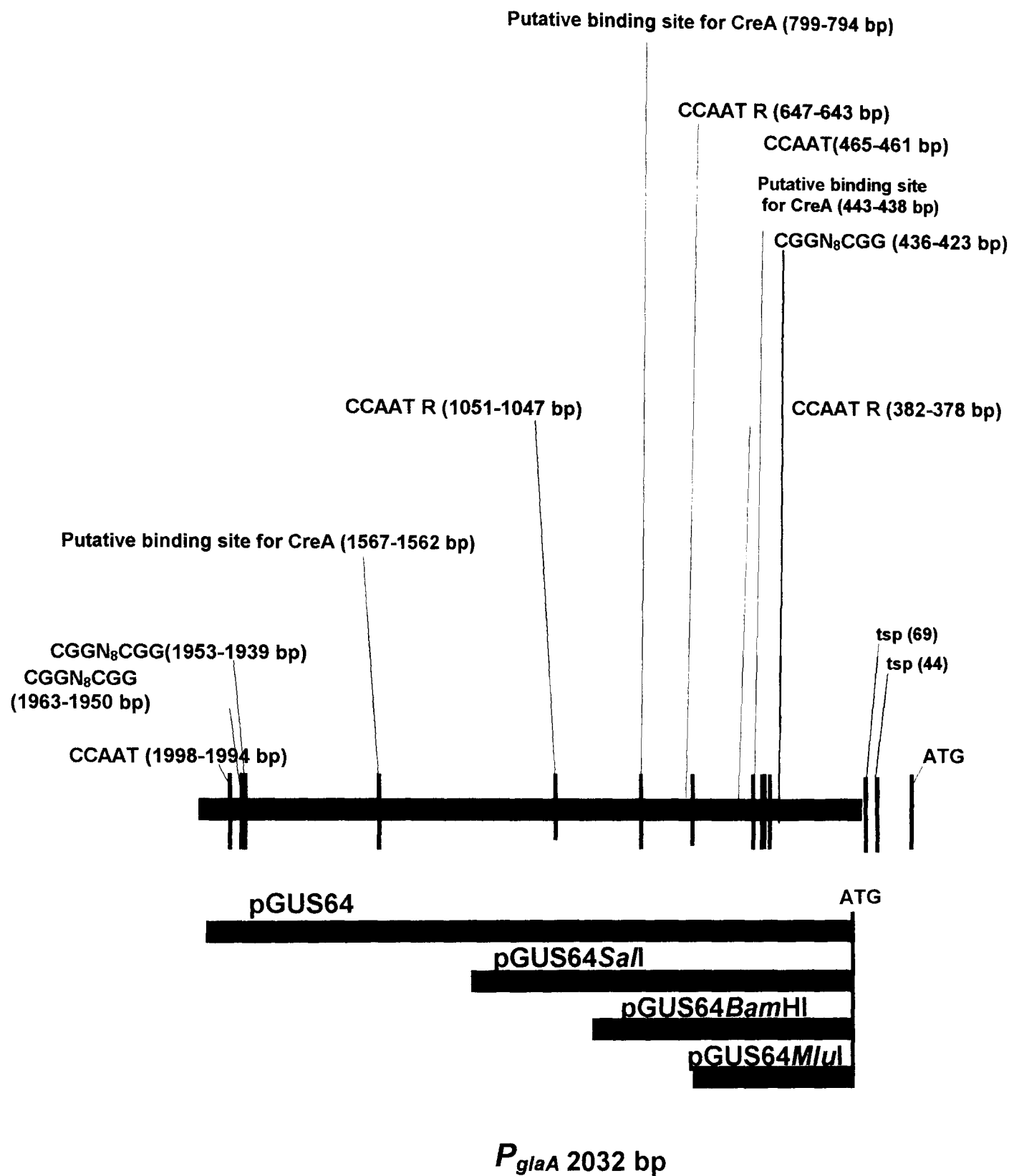
Filamentous fungi, such as *A. niger*, are widely used in the biotechnology industries to produce enzymes for a variety of uses (Archer and Peberdy, 1997). Expression of recombinant proteins may subject the fungus to ER stress that could activate the UPR and consequently limit protein secretion (Mulder *et al.*, 2004; Pakula *et al.*, 2003). One strategy used to improve the expression of recombinant proteins is to express the target gene under the control of a highly inducible endogenous promoter such as glucoamylase promoter (P_{glaA}). The starch-inducible P_{glaA} from *A. niger* is commonly used for this purpose (Table 1.4).

To facilitate the analysis of both gene expression and promoter roles under specific conditions reporter genes are widely used as tools for such purposes (Martin *et al.*, 1992; Santi *et al.*, 2003). The *E. coli uidA* reporter gene, which encodes GUS, has been extensively used in plants (Martin *et al.*, 1992) and in filamentous fungi (Abcede and Barraquio, 2003; Alcocer *et al.*, 2003; Brown *et al.*, 2003; Cary *et al.*, 2000; Fan *et al.*, 2001; Freeman *et al.*, 2002; Hata and Ishida, 2000; Ishida *et al.*, 2000; Toda *et al.*, 2001). The *E. coli* GUS enzyme (EC 3.2.1.31) is an acid hydrolase that catalyses the cleavage of a wide variety of β -glucuronides to their respective alcohol and glucuronic acid. The use of the *E. coli uidA* reporter gene system might be favourable for many reasons: GUS has high stability, many GUS substrates are water-soluble and commercially available (including substrates for spectrophotometric and fluorimetric analyses), the high sensitivity of the enzymatic assay and the possibility of obtaining both qualitative (histochemical) and quantitative (fluorimetric) data (Jefferson *et al.*, 1987), using, for example, the substrate 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-gluc) and 4-methylumbelliferyl β -D-glucuronide (MUG), respectively.

The reducing agent DTT induces the UPR in fungi and, in addition, the transcript level from *glaA* was lowered whereas the transcript level from the actin-encoding gene was not. To investigate this phenomenon more closely, analysis of

the P_{glaA} was carried out, to dissect its role under ER stress conditions. In the literature, several studies have characterised the regulation of P_{glaA} under different carbon sources. The molecular mechanism of P_{glaA} regulation in *A. niger* and *A. oryzae* was generally investigated by deletion analysis of P_{glaA} , often fused to a reporter gene or by introducing sequences believed to contain binding sites for positive transcriptional factors (Fowler *et al.*, 1990; Hata *et al.*, 1998; Henriksen *et al.*, 1999; Liu *et al.*, 2003; Verdoes *et al.*, 1994). Deletion analysis of *A. niger* P_{glaA} (815 bp) using *E. coli uidA* encoding-gene in reporter constructs, each introduced as a single copy at the same locus in the different transformants, was reported by Verdoes *et al.* (1994). In their study it was found that the largest reduction in the expression of the reporter gene took place when the upstream region of P_{glaA} was reduced from 815 bp to 522 bp. Thus, it was concluded that sequences between 815 bp and 522 bp are important for high-level expression. In contrast, Fowler *et al.* (1990) have previously reported the deletion analysis of 1966 bp of the sequence upstream of the translation start of a different strain of *A. niger*. They concluded that a region between 561 bp and 318 bp upstream of the *glaA* translational start is responsible for high-level expression. Their deletion study was complicated by the use of multicopy transformants, with their constructs integrated at various genomic loci. Although these studies have not been able to pinpoint regions of P_{glaA} responsible for induction by starch (or other compounds) or repression by xylose or glucose, later studies have defined regions for binding AmyR, a regulator (Gomi *et al.*, 2000; Petersen *et al.*, 1999). It has also been shown that the CCAAT sequence plays an important role in the transcriptional regulation of the *glaA* gene (Zhu *et al.*, 2004). Several putative binding sites for a protein that mediates carbon catabolite repression, CreA, (Drysdale *et al.*, 1993; Kulmburg *et al.*, 1993), the AmyR-binding site and the CCAAT sequence, are shown in Figure 4.1.

Figure 4.1 An illustrative map presents various features of *A. niger* P_{glaA} . Putative regulation sites for binding CreA (Kulmburg *et al.*, 1993) and AmyR (Petersen *et al.*, 1999) as well as CCAAT and reverse CCAAT proofs are shown. Transcriptional start points (tsp) as described by Boel *et al.* (1984) are also shown. Numbers shown are ascendant of the A nucleotide in the translation start codon ATG where the first nucleotide upstream of the translational start codon is +1. The N letter refers to any nucleotide (A,T,G,C) and the subscript number next to it refers to the number of nucleotides. The R letter indicates that the sequence is present in the reverse orientation.



A gene (*amyR*) encoding the transcriptional activator for amylase synthesis has been cloned from *A. oryzae* (Gomi *et al.*, 2000; Petersen *et al.*, 1999) and *A. nidulans* (Tani *et al.*, 2001b). The gene *amyR* encodes a transcriptional activator of 604 and 662 amino acid residues in *A. oryzae* and *A. nidulans*, respectively. The AmyR proteins in both species have a 100% conserved cys-6 zinc cluster DNA-binding motif at the NH₂-terminus, indicating that all AmyRs bind to identical DNA sequence motifs (Tani *et al.*, 2001a). The AmyR binds to two types of sequence, CGGN₈CGG and CGGAAATTAA, where N refers to any nucleotide (Petersen *et al.*, 1999). Disruption of *amyR* in *A. oryzae* transformant strains resulted in significantly poor growth on a starch medium, but on a glucose medium the growth was indistinguishable between these transformant strains (Gomi *et al.*, 2000). These observations on the effect of disruption of the *amyR* gene demonstrate the function of AmyR as a transcription activator for the amylase gene. The DNA sequencing of *P_{glaA}* used in this study showed three putative sites for binding AmyR, two of which overlap and are located 1939-1953 bp and 1950-1963 upstream of the translational start codon (Figure 4.1). In a previous study it was reported that deletion of the *P_{glaA}* region that contains these putative AmyR binding-sites had only a moderate effect on the expression of the *uidA* reporter (Verdoes *et al.*, 1994).

The regulation of *Aspergillus* genes encoding different amylases, especially glucoamylase, is controlled primarily at the transcriptional level. However, the level of gene expression can be affected by mRNA stability. The mRNA stability involved in the regulation of gene expression has been reported in mammalian cells (Guhaniyogi and Brewer, 2001), yeast (Heikkinen *et al.*, 2003) and filamentous fungi (Sachs, 1998). The mRNA stability can be affected at the transcriptional or at the post-transcriptional level. At the transcriptional level at least two factors can contribute to low levels of mRNA: 1 - a low mRNA stability and 2- incorrect processing of the transcripts. For example, incorrect processing of the pre-mRNA of a heterologous gene could take place due to the presence of an AU-rich sequence. That has been reported in *S. cerevisiae* overexpressing

Clostridium tetani tetanus toxin fragment (Romanos *et al.*, 1991) and HIV1-envelope protein in *Pichia pastoris* (Scorer *et al.*, 1993). Moreover, truncation in *aglA*, α -galactosidase-encoding gene, has been observed in *A. niger* and *A. nidulans*, which could possibly be due to the presence of an AU-rich sequence (Gouka *et al.*, 1996a). Recently, a specialised mRNA-decay pathway was discovered, termed the initiation-mediated decay pathway, that affects heat-shock mRNAs through decapping and 5'-to-3' hydrolysis, whereas a variety of non-heat shock mRNAs are not affected (Heikkinen *et al.*, 2003). The previously mentioned study has shown that five members of the yeast Hsp70 gene family *Ssa1*, *Ssa2*, *Ssa3*, *Ssc1*, and *Kar2*, and the two members of the Hsp90 gene family, *Hsp82* and *Hsc82*, were all subjected to initiation-mediated decay. These findings may suggest that initiation-mediated mRNA decay may play an important physiological role under stress conditions that require the involvement of heat shock proteins. However, pathways for mRNA degradation have not yet been studied extensively in filamentous fungi. Thus, it might be important when studying or characterising a feedback mechanism that is believed to operate at the transcriptional level to examine some mRNA feature such as size to ensure, at least initially, that truncation is not responsible for the decline in the transcripts level under the given growth conditions (i.e. exposure to DTT). This could initially be checked using some sensitive technique such as RT-PCR.

The expression of some recombinant proteins may be affected by ER conditions. For example, the production of the heterologous protein t-PA in the filamentous fungi *A. niger* and *T. reesei* results in *hacA* mRNA splicing (Mulder *et al.*, 2004; Saloheimo, personal communication). However, the function P_{glA} under ER stress, have not been studied.

In this Chapter the *uidA* expression level in *A. niger* reporter strains, grown in different carbon sources, was determined. Next, the role of P_{glA} in these reporter strains was studied under ER stress triggered by DTT. Finally, the stability of *uidA* mRNA under DTT treatment was checked by RPA and RT-PCR, and the transcriptional start point of *glA* was initially confirmed by RT-PCR.

4.2 Materials and Methods

4.2.1 *A. niger* reporter strains with different P_{glaA} truncation

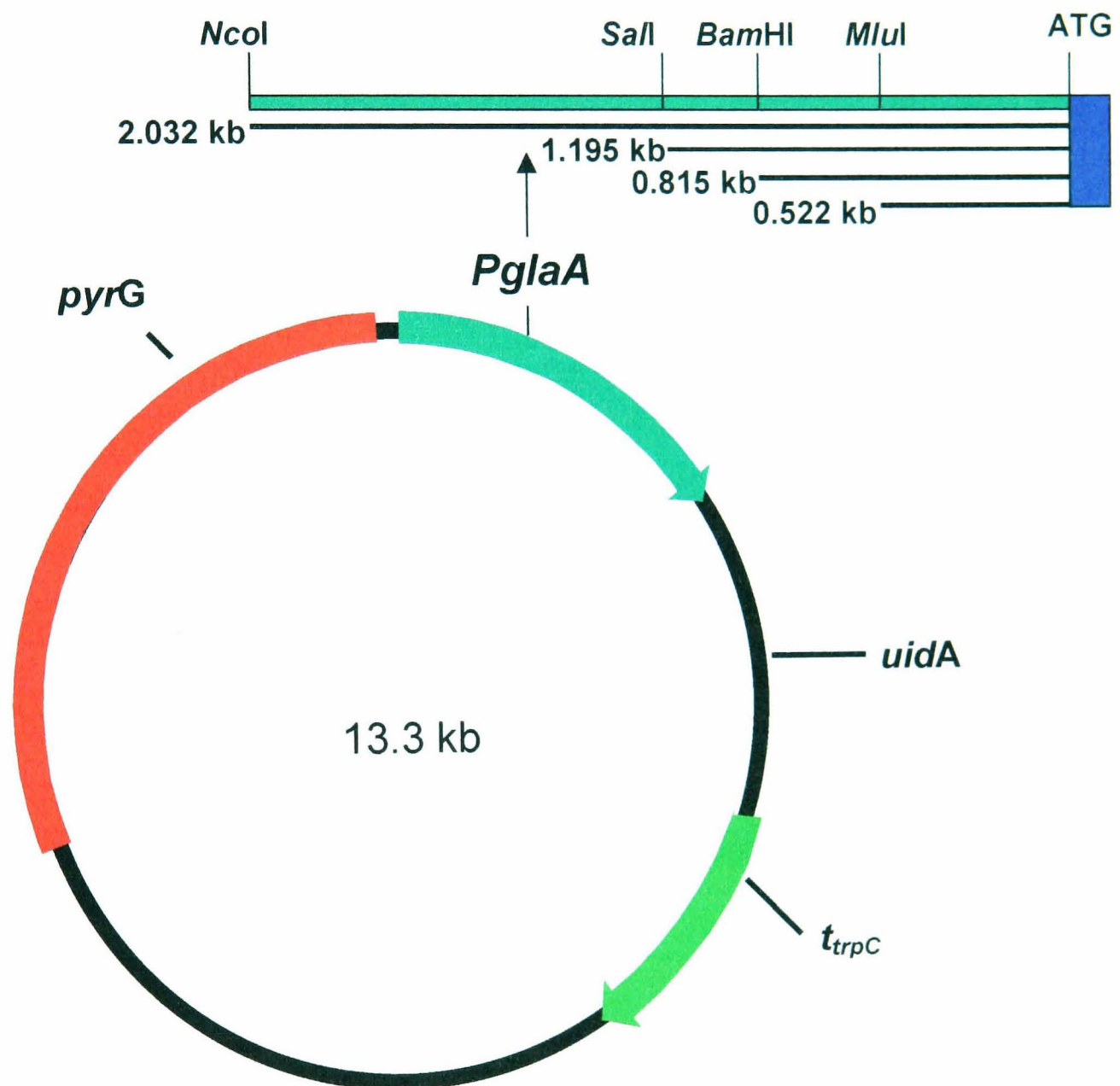
A. niger reporter strains used in this study to facilitate the analysis of P_{glaA} for the presence of sequences that are involved in mediating the decline of *glaA* transcripts under ER stress conditions were kindly provided by Dr. Punt (TNO Nutrition and Food Research Institute, The Netherlands.). Each of these reporter strains carries a reporter cassette consisting of a single copy of the *uidA* reporter gene under the control of the *A. niger* glucoamylase promoter, as illustrated in Figure 4.2. The *uidA* gene was placed under the control of approximately 2.032 kb, or after digesting with *Sall*, *Bam*HI, or *Mlu*I 1.195, 0.815, 0.522 kb P_{glaA} of *A. niger*, respectively. The plasmid constructs were termed pGUS64 (2.032 kb glucoamylase promoter (P_{glaA}), pGUS64 Δ S (1.195 kb P_{glaA}), pGUS64 Δ B (0.815 kb P_{glaA}), and pGUS64 Δ M (0.522 kb P_{glaA}) and were integrated as single-copies at the *pyrG* locus of *A. niger*. Thus, the *A. niger* reporter strains are referred to in this study as GUS64, GUS64*Sall*, GUS64*Bam*HI, and GUS64*Mlu*I, respectively (Verdoes *et al.*, 1994).

4.2.2 RT-PCR primers

RT-PCR was carried out as described in Chapter 3 section 3.2.2 using the following primer sets: Set I primers were the forward primer uidHA01F (CCT CTA GAA AGC GTA TGT TAC GTC CTG TGA AAA CCC CA), and the reverse primer uidHA02R (CAC CAC CTG CCA GTC AAC A) and the annealing temperature was 58°C. Set II primers were the forward primer P2F (CGT GAG GGG CTG AAG TGC T) and the reverse primer uidHA03R (CCG TAA TGA GTG ACC GCA TCG) and the annealing at 58°C.

Figure 4.2 Plasmid vectors used for the construction of the reporter strains. Thick lines represent *Aspergillus* DNA and thin lines represent *E. coli* DNA. Construction details are described in Verdoes *et al.* (1994). A mutated *pyrG* gene (*pyrG**) was used to facilitate the selection of the reporter construct integration at the *pyrG* locus as a single copy. The terminator used was the *trpC* (*t_{trpC}*) of *A. nidulans*. Restriction enzymes used for the deletion analysis of *P_{glaA}* are shown.

Single copy integrated vector at the *pyrG* locus of *A. niger*



4.2.3 Preparation of DNA probes for Ribonuclease Protection Assay (RPA)

RPA probes for actin and *bipA* were prepared by first cloning the PCR-amplified probes, using primers described in Table 3.1, into the pGEM-T Easy vector (Promega, USA), except for the *uidA* probe. The *uidA* probe was a 552bp fragment corresponding to co-ordinates +1261 to +1812 in the sequence of *uidA* and it was amplified by PCR with the primers *uidA*^{#a}FW (5'AATATTTTCGCGCCCACTGG^{3'}), *uidA*^{#a}REV (5'TCTAGATCATTGTTTGCCTC^{3'}). The cloned probe was checked by endonuclease restriction digests. After confirming the cloning of the target gene, the plasmid DNA (pGEM-T Easy vector) (12µg) was linearised with a restriction enzyme that cuts in a site that allows the complementary mRNA (antisense) transcripts to be synthesized for the desired length of RNA transcripts from the T7 or SP6 RNA polymerase promoter present in the pGEM-T Easy vector, as illustrated in Figure 4.3. RNA transcripts which were used as RPA probes were prepared using the MAXIscript® SP6/T7 Kit (Ambion, USA) following the manufacturer's instructions. After linearisation, the DNA template was cleaned by treating with 30µl Proteinase K (200 µg/ml) and 5µl SDS (10%) for 30 minutes in 50°C, followed by phenol/chloroform extraction and ethanol precipitation. The cleaned DNA template was dissolved in 30µl RNase-free water and the concentration was determined spectrophotometrically as described in Chapter 2. The *in vitro* transcription reaction was then carried out using T7 or SP6 enzyme, following the manufacturer's instructions (Ambion, USA). After the transcription reaction, each probe was diluted ten fold in RNase-free water before performing the Radiolabel Incorporation test for synthesized RNA probes (below).

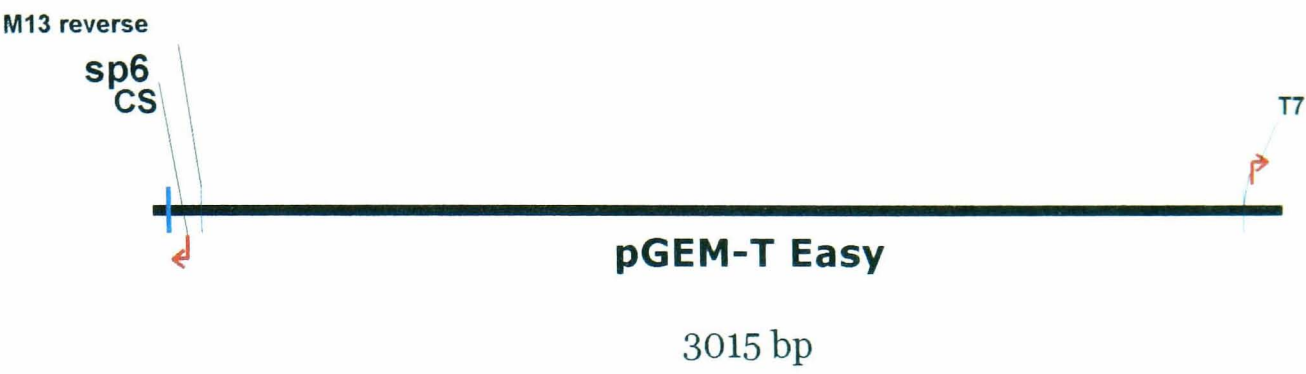
4.2.4 Radiolabel Incorporation test for synthesized RNA probes

The amount of radiolabel (α -³²P UTP) incorporated into the transcript product was determined as follows: 1µl of the diluted probe was spotted into the middle of DE81 paper and left to dry for a few minutes.

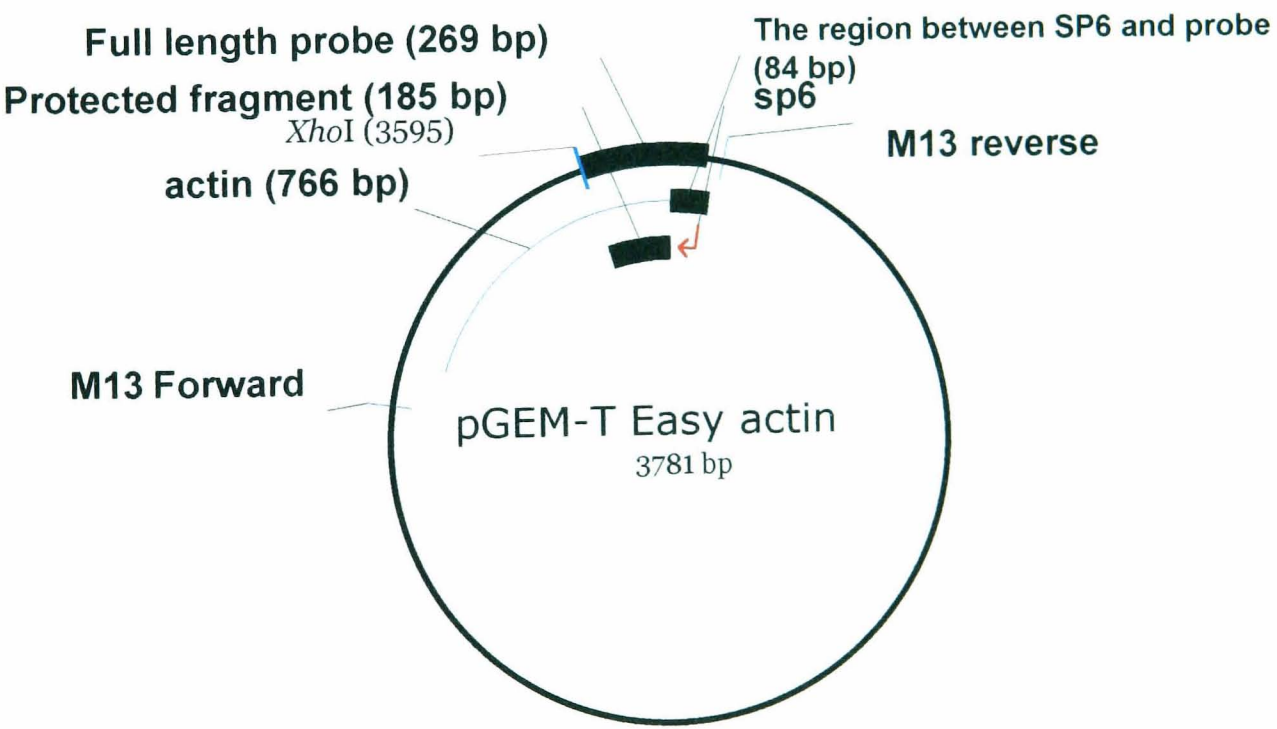
Figure 4.3 Schematic diagram of the pGEM-T Easy plasmid vector used to clone the RPA probes. The size of each cloned probe and the size of the protected fragment are shown. The restriction enzymes used to linearise the plasmid vector and facilitate transcription from the desired T7 or SP6 promoters are shown.

- a. Schematic diagram of pGEM-T Easy plasmid vector (template)
- b. Schematic diagram of pGEM-T Easy plasmid vector after cloning the actin probe
- c. Schematic diagram of pGEM-T Easy plasmid vector after cloning the *bipA* probe
- d. Schematic diagram of pGEM-T Easy plasmid vector after cloning the *uidA* probe

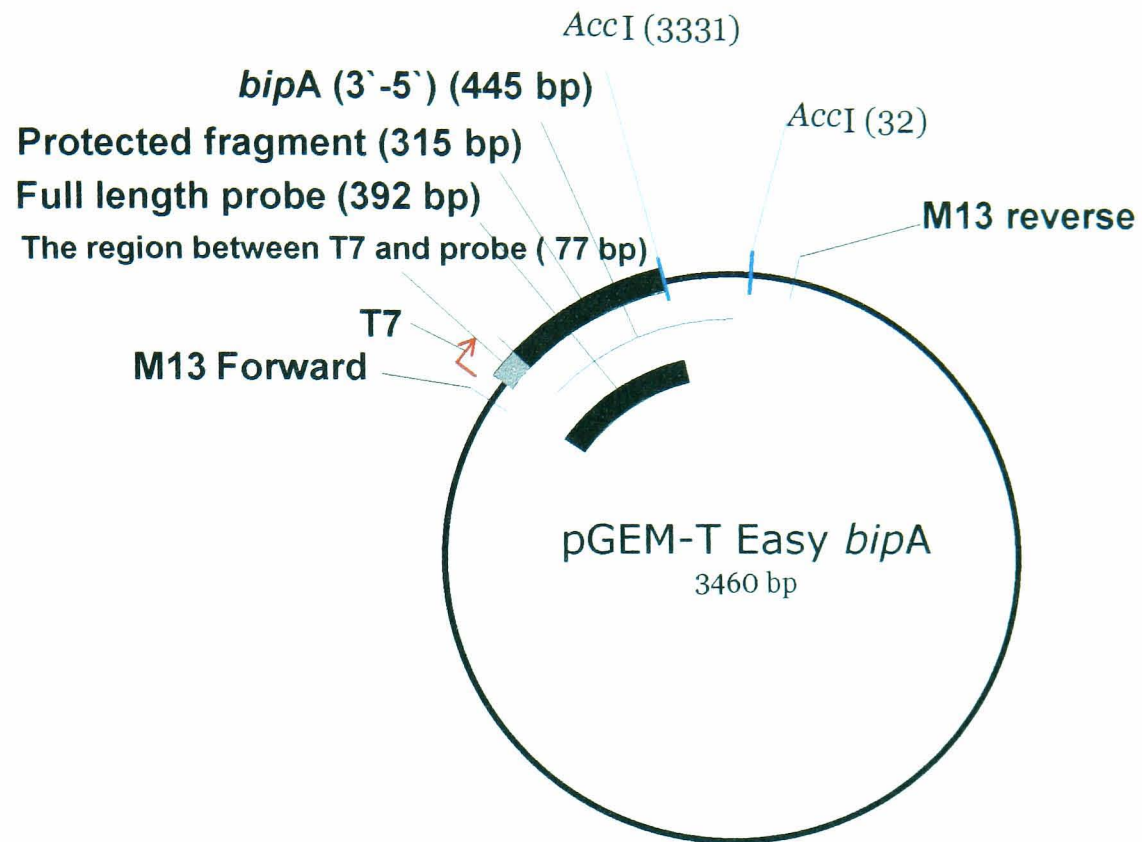
a.



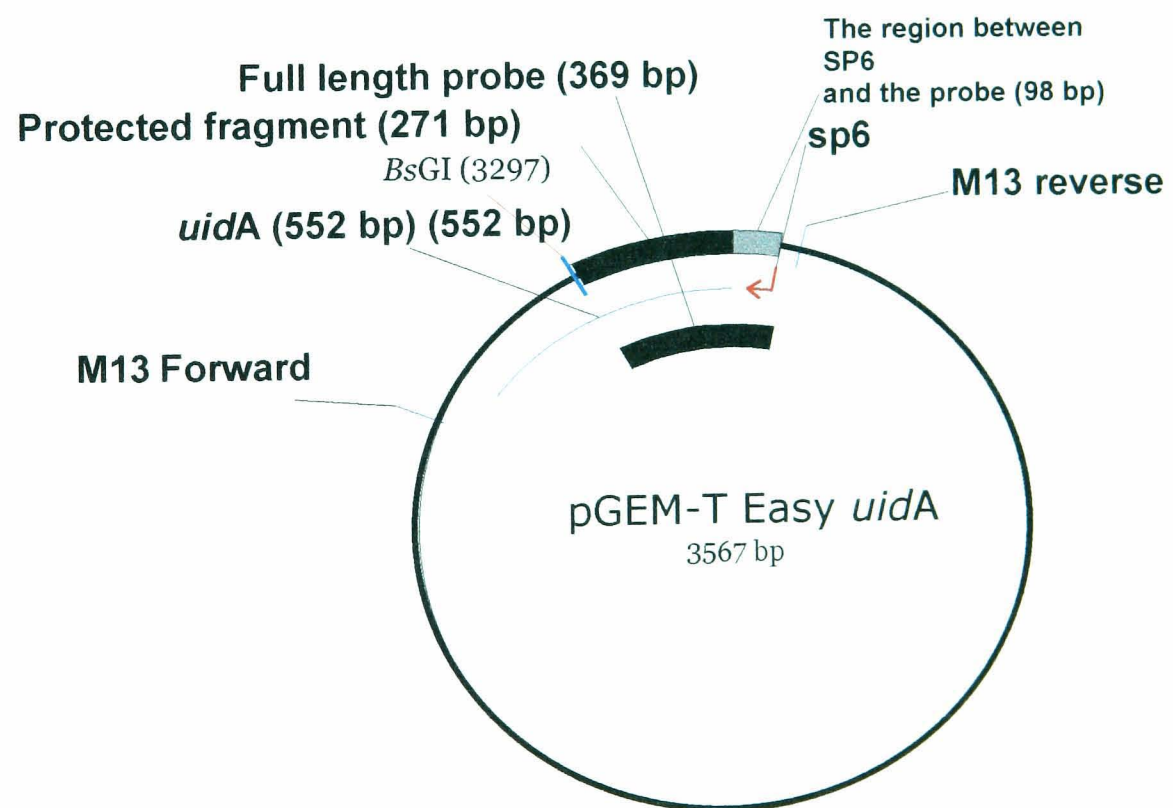
b.



c.



d.



The radioactivity in the DE81 paper was estimated using a Geiger counter, and the DE81 paper was washed four times (5 minutes each) in 0.5M Na₂HPO₄ followed by one wash for 2 minutes in H₂O to remove the salt. The DE81 paper was then soaked for 2 minutes in ethanol to remove the water and then left to dry for 10-15 minutes, after which the radioactivity was re-measured to find out the level (percent) of α -³²P UTP incorporation. After the incorporation test, each probe was diluted 1/10, 1/100, or 1/1000 as required (Figure 4.6). The incorporation test allows a constant addition of the probe each time the RPA assay is carried out, as the incorporation level may vary.

4.2.5 Ribonuclease Protection Assay (RPA)

The RPA was carried out using the RPAIII kit (Ambion, USA) following the manufacturer's instructions and using 10 μ g of total RNA per reaction. The total RNA concentration per reaction was determined empirically to be enough to detect the target mRNA while the labeled probe was present in at least 10 fold excess over the target mRNA (Figure 4.6).

4.2.6 Preparation of and running the denaturing polyacrylamide gels

Final RPA samples were run on 5% (w/v) acrylamide TBE gels prepared as follows. 28.8 g of high quality urea was dissolved in 6 ml of 10X Tris borate EDTA (TBE) (109 g/l Tris base, 55 g/l boric acid, and 40 ml of 0.5 M EDTA solution), 7.6 ml of 40% acrylamide/bis-acrylamide, and 60 ml distilled H₂O. The mixture was stirred at room temperature until the urea had dissolved, and then de-gassed for 5 minutes. Next, 500 μ l of 10% (w/v) ammonium persulfate and 65 μ l TEMED were added, which catalyses the polymerization, and the mixture was immediately poured. Prior to loading the sample, the urea was rinsed out of the wells, and samples were electrophoresed against RNA Century markers (Ambion) at 200V for 2 hours.

4.2.7 Testing for DTT interference with the fluorimetric GUS assay

Mycelial extracts were prepared as outlined in Chapter 2 section 2.10, from GUS64 strain grown in ACMS/N/P for 48 hours with no addition of DTT. The

mycelial extracts were then divided into aliquots. Next, DTT was added and the GUS assay was carried out immediately, and at 3, and 6 hours after DTT addition to the supernatant at 20 mM final concentration. To test the DTT effects on the activity of GUS (Sigma) and on the fluorescence activity of the calibration standard, DTT was added to the reaction mix prepared as described in Chapter 2 and to a series of diluted 4-methylumbelliferone (Sigma; free acid) solutions, standard for calibration, respectively. The fluorescence activities from these samples were then detected fluorimetrically as described in Chapter 2.

4.3 Results

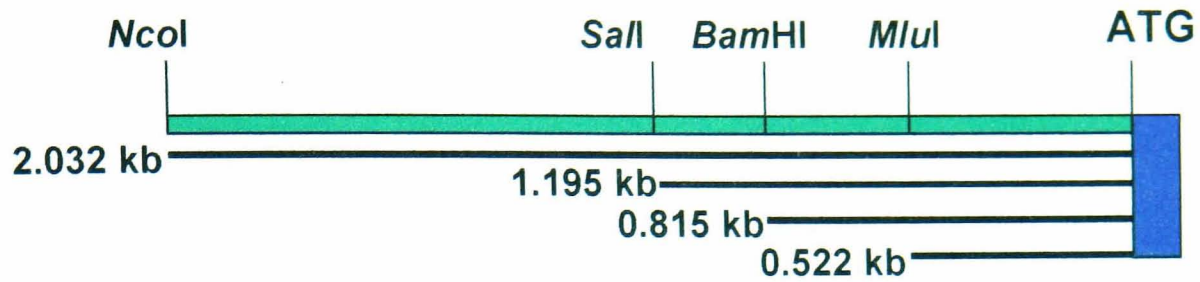
4.3.1 DNA sequence of the truncated glucoamylase promoter (P_{glaA}) used in the *A. niger* reporter strains

Truncated P_{glaA} regions used in the construction of the reporter cassettes shown in Figure 4.2 were further sequenced at the University of Nottingham. These sequences were kindly provided by Dr. Alcocer and are shown in Figure 4.4. Vector NTI computer software (InforMax, Inc., UK) was used to analyse these sequences. Several putative sites that were reported previously (CreA-binding and CCAAT) or those pointed out in this study (CCAAT (upstream 1kb) and AmyR-binding) are shown in Figure 4.1.

4.3.2 The *uidA* expression level in *A. niger* reporter strains GUS64, GUS64*Sa*II, GUS64*Bam*HI, and GUS64*Mlu*I grown in different carbon sources

Figure 4.5 shows the *uidA* expression levels in *A. niger* reporter strains GUS64, GUS64*Sa*II, GUS64*Bam*HI, and GUS64*Mlu*I grown in ACM/N/P medium containing starch, glucose or xylose as the carbon source. Previously, Verdoes *et al.* (1994) used the same reporter strains to perform a deletion analysis of P_{glaA} . However, in their study maltose was used instead of starch as the carbon source to induce P_{glaA} . The advantage of having these reporter constructs integrated at the same locus (Figure 4.2) as a single copy is that any variation in the transcriptional level of the reporter gene between these transformants, due to the locus of integration in the genome, is avoided.

Figure 4.4 The full length (2032 bp) of the *P_{glaA}* is shown. The top diagram shows the size of each *P_{glaA}* truncated fragment and the restriction enzymes used for the construction of these truncated fragments. The restriction sites (nt sequences) for these enzymes are indicated in bold in the nt sequence of *P_{glaA}*. Size shown for each truncated fragment refers to the size from the first nucleotide in the fragment up to the translation start codon (ATG) of the reporter gene, which is shown in capital letters. For example, the pGUS64 uses the 2032 bp promoter that begins with tcct and continues up to the translation start codon (ATG) of the reporter gene.



```

1  tcctcgtga tctcccttg gtacagtcgg ctaccaatgc tctcgaggat tgcctgaaca
61  ttgacattcg gcgtccggcc gggaccaccg cggactcgaa gctgcctgtg ctggtctgga
121 tctttggcgg aggctttgaa cttggttcaa aggcgatgta tgatggtaca gcgatggtat
181 catcgtcgat agacaagaac atgcctatcg tgtttgtagc aatgaattat cgcgtgggag
241 gtttcggggtt cttgcccgga aaggagatcc tggaggacgg gtccgcgaac ctagggtccc
301 tggaccaacg ccttgccctg cagtgggttg ccgacaacat cgaggccttt ggtggagacc
361 cggacaaggt gacgatttgg ggagaatcaa gcaggagcca tttccgtttt tgatcagatg
421 atcttgtacg acggaaacat cacttacaag gataagccct tgttcggggg ggccatcatg
481 gactccggta gtgttgaacc cgcagacccc gtcgatgggg tcaagggaca gcaagtatat
541 gatgcggtag tggaatctgc aggctgntcc tcttctaacg acaccctagc ttgtctgcgt
601 gaactagact acaccgactt cctcaatgcg gcaaactccg tgccaggcat tcaagctacc
661 attctgtggc cgttatcata tgtgcctcga ccggacggga cggcgttgtc ggcacaccg
721 gacgttttgg gcaaagcagg gaaatatgct cgggtcccgt tcatcgtggg cgaccaagag
781 gatgagggga ccttattcgc cttgtttcag tccaacatta cgacgatcga cgaggtgsallgtc
841 gactacctgg cctcatactt cttctatgac gctagccgag agcagcttga agaactagtg
901 gccctgtacc cagacaccac cacgtacggg tctccgttca ggacaggcgc ggccaacaac
961 tggtatccgc aatttaagcg attggccgcc attctcggcg acttggctct caccattacc
1021 cggcgggcat tcctctcgta tgcagaggaa atctcccctg atcttccgaa ctggtcgtac
1081 ctggcgacct atgactatgg caccacagtt ctggggacct tccacggaag tgacctgctg
1141 caggtgttct atgggatcaa gccaaactat gcagctagtt ctagccacac gtactatctg
1201 agctttgtgt atacgctBamHIgga tccgaactcc aaccgggggg agtacattga gtggccgcag
1261 tggaaggaat cgcggcagtt gatgaatttc ggagcgaacg acgccagtct ccttacggat
1321 gatttccgca acgggacata tgagttcatc ctgcagaata ccgcggcggt ccacatctga
1381 tgccattggc ggaggggtcc ggacggtcag gaacttagcc ttatgagatg aatgatggac
1441 gtgtctggcc tcggaaaagg atatatgggg atcataatag tactagccat attaataag
1501 ggcataatac MluIacgcgttggga cctgcgttat agcttcccgt tagttatagt accatcgta
1561 taccagccaa tcaagtcacc acgcacgacc ggggacggcg aatccccggg aattgaaaga
1621 aattgcatcc taggccagtg aggccagcga ttggccacct ctccaagcac acagggccat
1681 tctgcagcgc tgggtggattc atcgcaattt cccccggccc ggcccagacac cgctatagga
1741 tggttctccc acaccatcgg agattcgtcg cctaattgtc ctgcgttca caagctgaag
1801 agcttgaagt ggcgagatgt ctctgcagga attcaagcta gatgctaagc gatattgcat
1861 ggcaatatgt gttgatgcat gtgcttcttc cttcagcttc ccctcgtgca gatgaggttt
1921 ggctataaat tgaagtgggt ggtcgggggt ccgtgagggg ctgaagtgct tcctcccttt
1981 tagacgcaac tgagagcctg agcttcatcc ccagcatcat tacacctcag ccATG

```

The DNA sequence of the truncated P_{glaA} in each of the reporter strains is shown in Figure 4.4. The highest level of GUS specific activity was produced by *A. niger* reporter strain GUS64 with the biggest P_{glaA} fragment of 2032 bp, as shown in Figure 4.5 (a). By comparing the *uidA* mRNA levels to the quantity of GUS activity detected in each reporter strain (Table 4.1), two main observations could be made. First, the promoter region between 1.195 and 2.032 kb upstream of the translation start contains some promoter element(s) important for transcription. Second, the minimum size of P_{glaA} required for low-level expression appears to lie within the first 522 bp upstream of the translation initiation site. This is supported by the observation that shortening the promoter further results in a complete loss of expression (Verdoes *et al.*, 1994). Interestingly, when these reporter strains were grown in ACM/N/P medium containing glucose as the sole carbon source, only the GUS64 strain, which has the biggest P_{glaA} fragment (2032 bp), produced a detectable level of GUS (Figure 4.5b). A simple explanation for this observation is that a promoter site(s) required for high-level expression in a glucose-containing medium is located within the 2000nt-1000nt P_{glaA} . No further investigation of this observation was carried out.

Figure 4.5 GUS activity in *A. niger* reporter strains grown in different carbon sources (final concentration of 1% w/v) for 48 hours. Experiments were carried out in 250 ml flasks each with 100 ml of medium. The GUS activity calculated represents the average \pm SEM of three replicates.

- a. GUS activity in *A. niger* reporter strains grown in different carbon sources (final concentration of 1% w/v)
- b. GUS activity in *A. niger* GUS64 strain grown in different carbon sources (final concentration of 1%)

In the Figure symbol legend, starch, glucose and xylose indicate the growth medium used, ACMS/N/P, ACMG/N/P and ACMX/N/P, respectively.

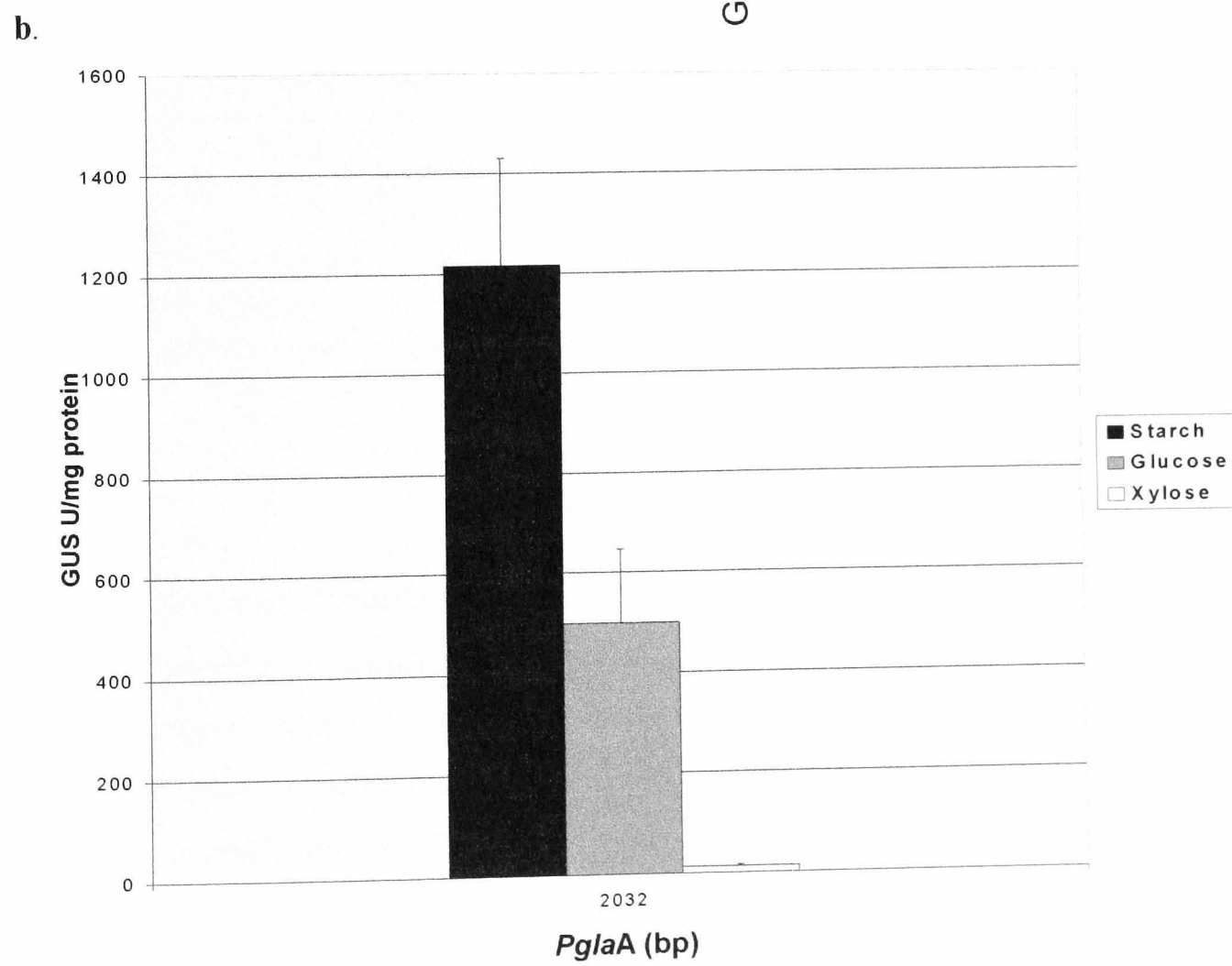
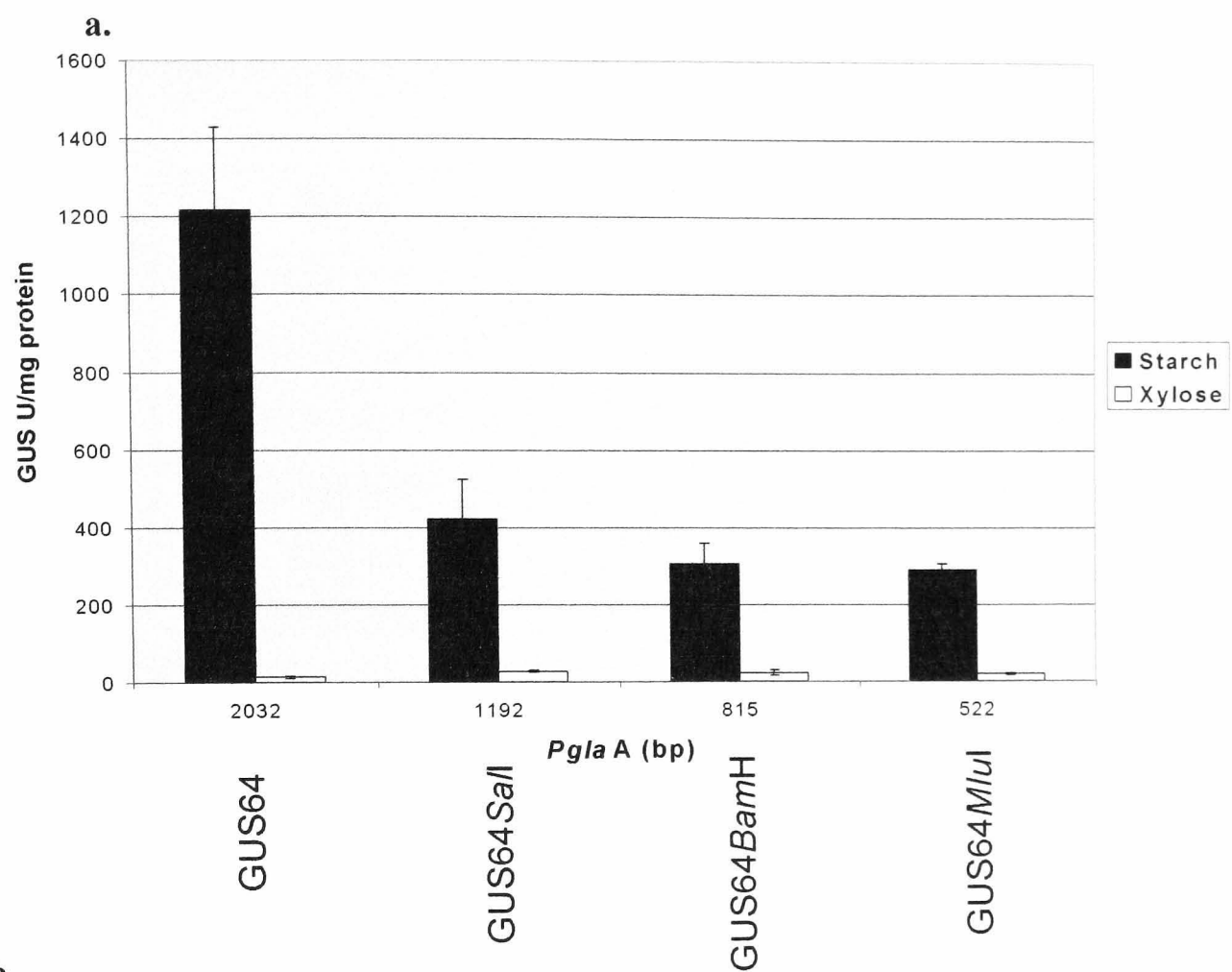


Table 4.1 Analysis of GUS activity in *A. niger* reporter strains: a summary of data presented in Figure 4.5

Strain	Carbon source	GUS activity (Gus U/mg protein)
GUS64	S	1215.7 (\pm 214.3)
GUS64 <i>Sa</i> II	S	421.3 (\pm 103.4)
GUS64 <i>Ba</i> mHI	S	303.9 (\pm 53.8)
GUS64 <i>Mi</i> uI	S	288.6 (\pm 16.5)
GUS64	G	498.7 (\pm 147.5)
GUS64 <i>Sa</i> II	G	<1
GUS64 <i>Ba</i> mHI	G	<1
GUS64 <i>Mi</i> uI	G	<1
GUS64	X	12.7 (\pm 3.8)
GUS64 <i>Sa</i> II	X	26.9 (\pm 2.3)
GUS64 <i>Ba</i> mHI	X	23.4 (\pm 6.8)
GUS64 <i>Mi</i> uI	X	17.8 (\pm 2.2)

- S, G, X, represent: starch, glucose, and xylose respectively, U: unit of GUS activity
- Numbers in parentheses are an average \pm SEM of three replicates

4.3.3 Positive and negative controls used in RPA showed the expected patterns

Two control probes were run in the RPA experiments. One control sample had no-RNase added - this control should show the full-length probe (positive control) - while the other control had no-RNA target/+RNase (negative control). In this negative control, mouse liver RNA (Ambion) was used instead of *A. niger* RNA. The no-RNA target/+RNase sample lane in the gel should show no signal. Figure

4.6 shows that the positive and negative controls gave the expected results. Figure 4.6 also confirms the optimal concentration (dilution) (1:100) of the RNase T1 (Ambion), which should not result in over- or under-digestion of the band. Also, Figure 4.6 shows different dilutions of the probe that were prepared prior to the hybridisation step. Detecting the optimal dilution of the probe is very important in the RPA, since a very concentrated probe could mask the protected band. Also, a low concentration of the probe could result in under-estimation of the target. The probe concentration was kept consistent in all RPA by carrying out the incorporation test as described in the Materials and Methods section 4.2.3.

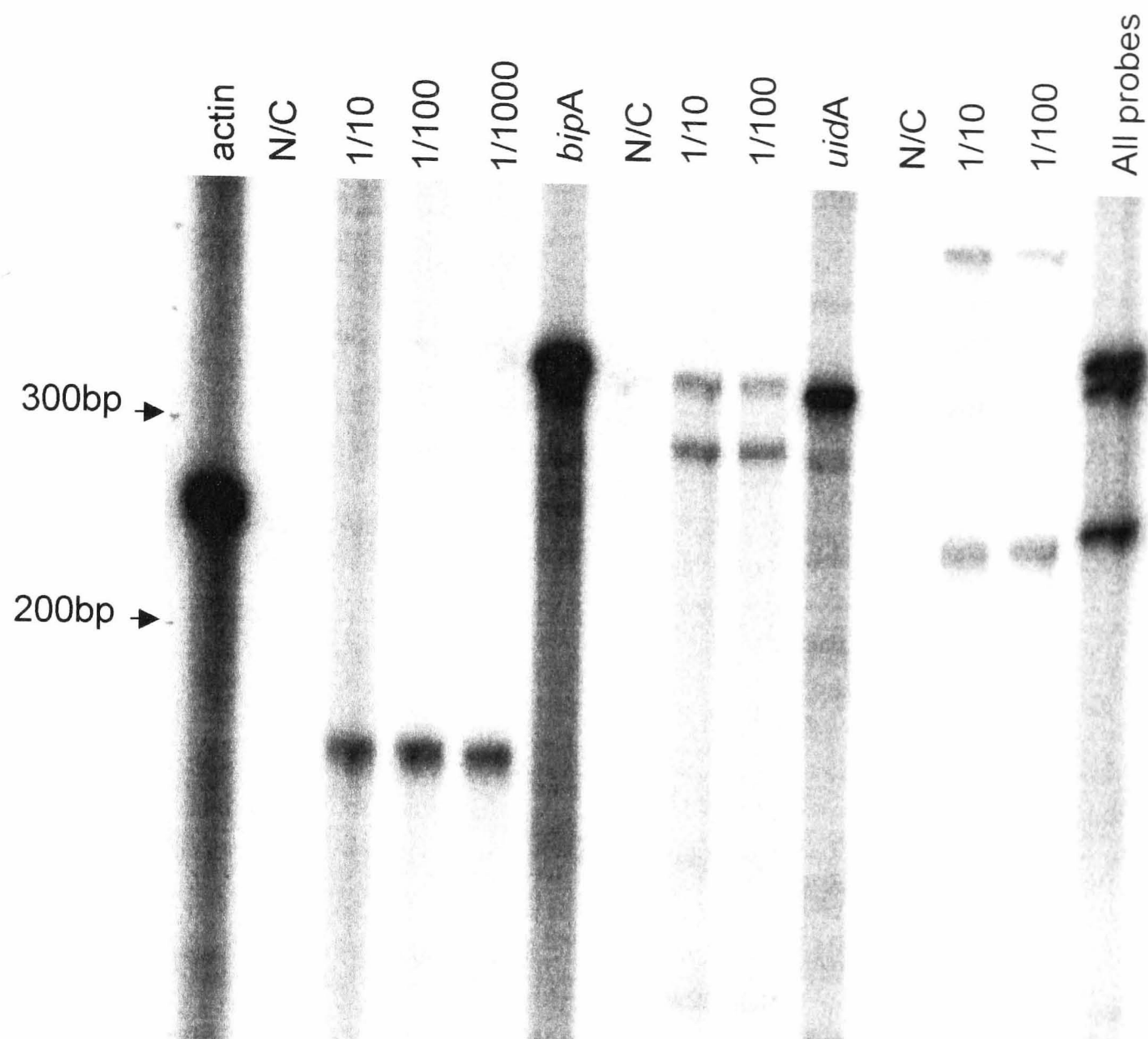


Figure 4.6 RPA probes for actin, *bipA*, and *uidA*. First lane of each probe shows the full length of the probe (no RNase added). Probes were diluted as indicated (by 1/10, 1/100, 1/1000) and hybridized to their targets. For the negative control (N/C), mouse liver RNA was used (10 μ g). The last lane shows all full length probes, but loaded at lower concentrations.

4.3.4 Effects of DTT treatments on the transcript levels of the *uidA* reporter gene and the gene-encoding the molecular chaperone *bipA* in *A. niger* reporter strains GUS64, GUS64*SaI*, GUS64*Bam*HI, and GUS64*Mlu*I

To study whether the decline of the steady state mRNA levels of the glucoamylase-encoding gene under ER stress (previous Chapter) is mediated by promoter elements present in P_{glaA} , *A. niger* reporter strains (Figure 4.2) were used. The ER stress was applied by treating these reporter strains with 20mM DTT (final concentration) and the steady state mRNA levels of the reporter strain were detected by RPA (data represented in Figure 4.7). Table 4.2 compares the *uidA* transcript level of the control samples as detected by RPA (Figure 4.7), with the corresponding level of GUS detected by GUS assay (Figure 4.5), where the relative *uidA* expression refers to that of pGUS64 as 100%. Although the accuracy of the correlation between the levels of *uidA* mRNA and β -glucuronidase (GUS) may depend on the sensitivity of the assays used to detect them, Table 4.2 generally illustrates the correlation of the mRNA and protein expression levels.

Another observation that can be made from Figure 4.7a is that, under ER stress triggered by DTT treatment, the decline of *uidA* mRNA only occurred when the *uidA* gene was expressed under the control of 2032 bp of P_{glaA} (strain GUS64). This is further illustrated by the number shown above the DTT treated sample bars which refer to the *uidA* mRNA level relative to t_{0h} (100%) of each sample. These numbers should show whether the *uidA* level is declining or not over the DTT six hours time-course used. It can be clearly seen that a decline in *uidA* mRNA level took place only when the *uidA* reporter gene was expressed under the control of 2032 bp P_{glaA} . This suggests that the decline resulted from transcriptional down-regulation by promoter elements present within a region of P_{glaA} located 1195-2032 bp upstream of the translational start.

To confirm that reporter strains were indeed under ER stress as a result of DTT treatment, the level of *bipA* transcripts was checked in the same mRNA

samples used from these reporter strains to check the *uidA* transcript level Figure 4.7 (a). As shown in Figure 4.7b, *bipA* was clearly induced in the DTT treated samples in all the reporter strains. The obvious *bipA* up-regulation should indicate UPR activation in these reporter strains. The average induction level of *bipA* was about five fold after 60 minutes of DTT treatment and about seven fold after 360 minutes of DTT treatment.

Interpreting the data in Figure 4.7 is complicated because steady-state levels of mRNA are measured. The relative effects of mRNA stability and new mRNA synthesis cannot be distinguished. Therefore, this analysis was taken further using medium-exchange studies coupled to RPA and nuclear run-on analysis (see Chapter 5).

Also checking the mRNA size at this stage might be interesting, especially in *A. niger* pGUS64 and pGUS64*Sa*II reporter strains under DTT treatment, since the stability of the mRNA could be affected by the mRNA truncation (Gouka *et al.*, 1997a). RT-PCR analysis was carried out later on in this Chapter to initially check this possibility of mRNA truncation under DTT treatment.

Table 4.2 The *uidA* expression levels in *A. niger* reporter strains grown in ACMS/N/P medium. The relative *uidA* expression refers to the activity of GUS64 (100%) measured in the same experiment. The *uidA* mRNA levels correspond to the GUS activities presented in Figure 4.5 and Table 4.1.

<i>A. niger</i> reporter strain	Relative <i>uidA</i> mRNA level at 48 hours of growth (%)	Relative GUS level at 48 hours of growth (%)
GUS64	100	100
GUS64 <i>Sa</i> I	57.8	34.7
GUS64 <i>Ba</i> mHI	52.9	25
GUS64 <i>Ml</i> uI	40.6	23.7

For the average *uidA* expression in GUS64 see Figures 4.5 and 4.7

Figure 4.7

a.

Effect of DTT stress on transcription of the *uidA* gene compared with water-treated control cultures of *A. niger* reporter strains. The graph shows the RPA of the *uidA* transcripts from *A. niger* reporter strains GUS64, GUS64*Sal*I, GUS64*Bam*HI, and GUS64*Mlu*I. The size of *P_{glaA}* in each reporter strain is shown. The levels of *uidA* transcripts were normalised against the corresponding γ -actin control and the values for each time-point calculated relative to the t_{0h} value (taken as 100%). The numbers shown above the DTT-treated sample bars (white) refer to the *uidA* mRNA level of t_{0h} (100%) of each sample. The water-treated samples (referred to as control in the graph) are represented by black columns. The t_{0h} points, which have a value of 1, are not shown in the graph. The *uidA* expression levels are an average \pm SEM of three replicates.

a.

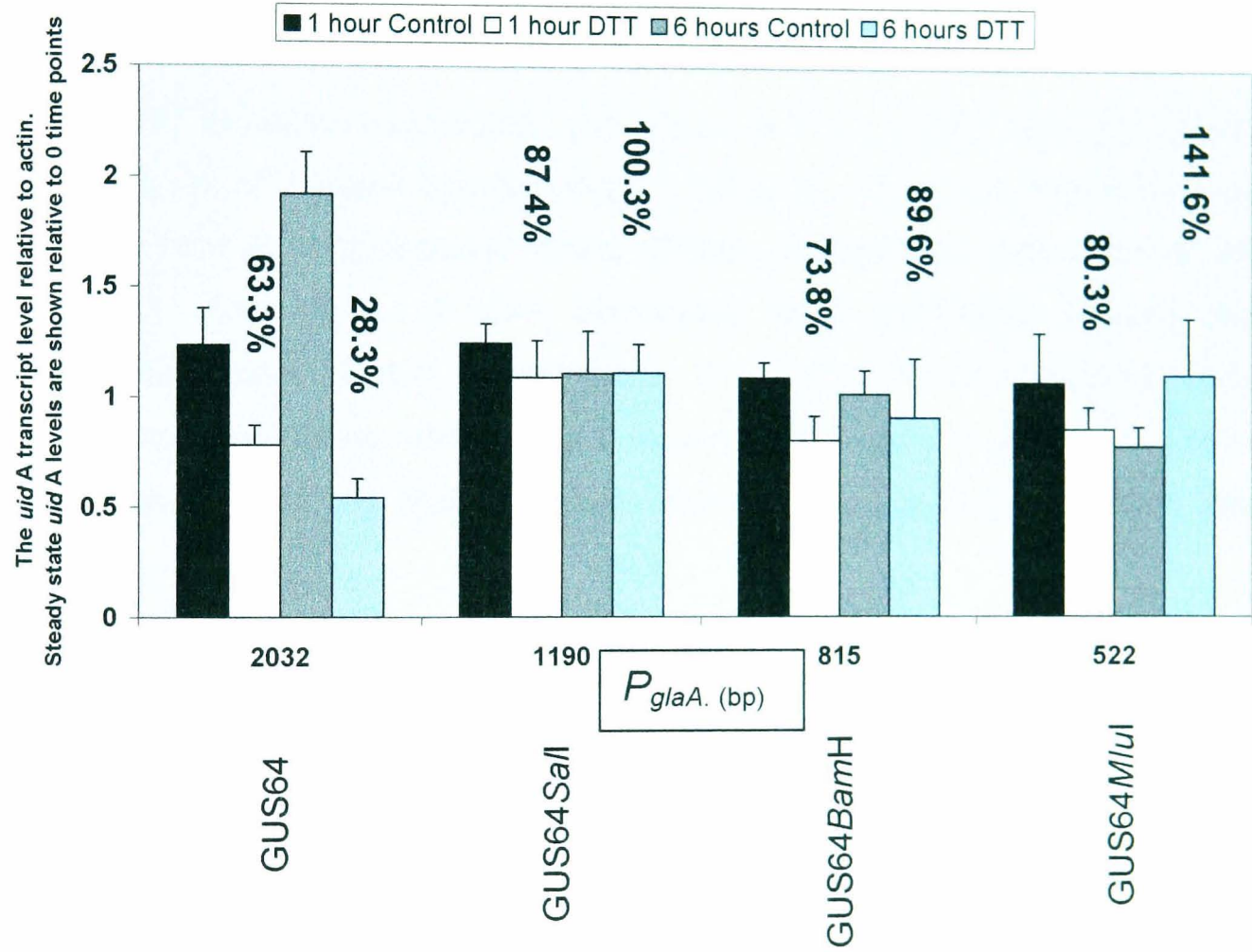


Figure 4.7

b.

Effect of DTT stress on transcription of the *bipA* gene compared with water-treated control cultures of *A. niger* reporter strains. The graph shows the RPA the of *bipA* transcripts from *A. niger* reporter strains GUS64, GUS64*Sal*I, GUS64*Bam*HI, and GUS64*Mlu*I. The levels of *bipA* transcripts were normalised against their corresponding γ -actin control and the values for each time-point calculated relative to the t_{0h} value (taken as 100%). The t_{0h} points, which have a value of 1, are not shown in the graph. The *bipA* expression levels are an average \pm SEM of three replicates.

b.

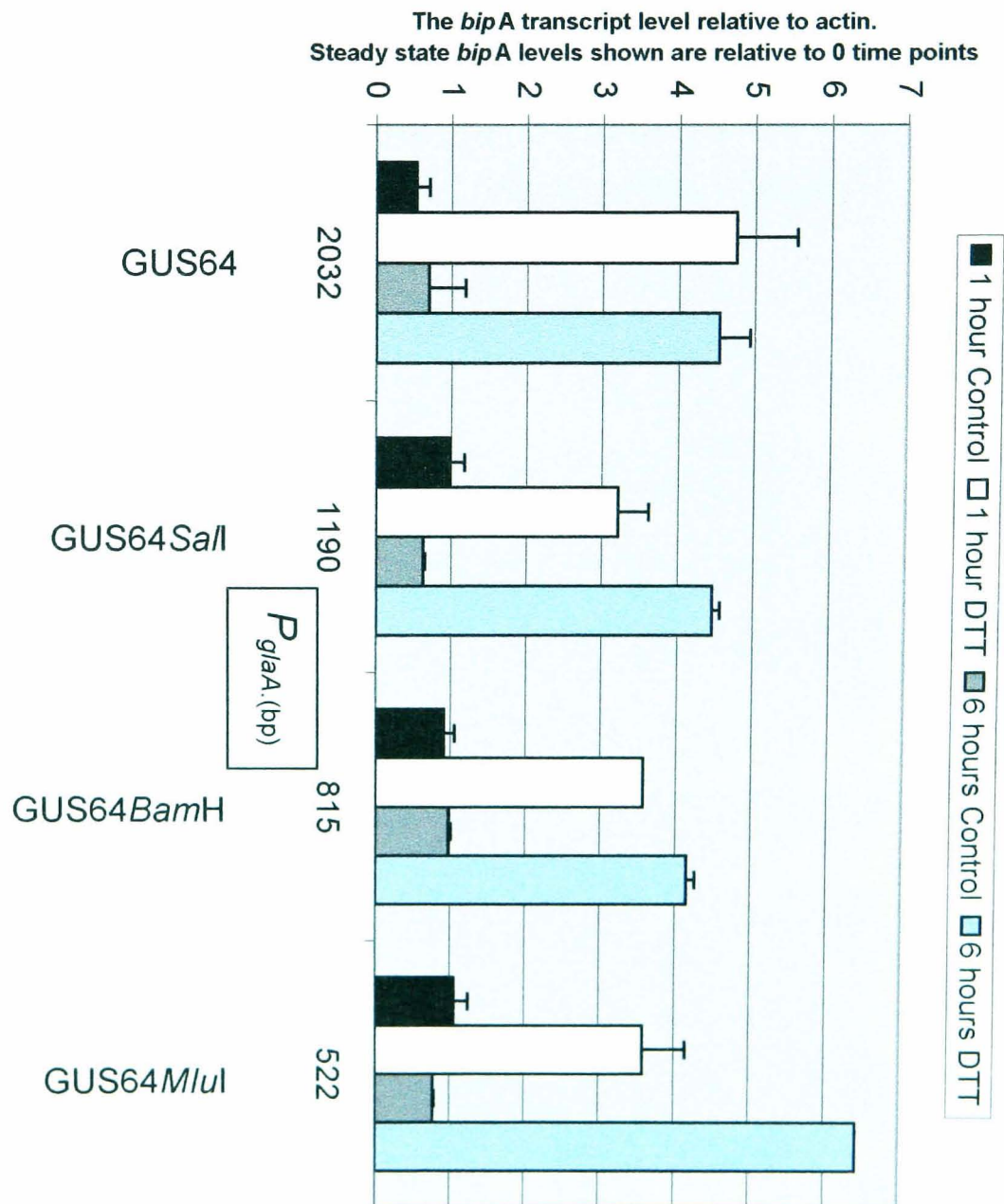
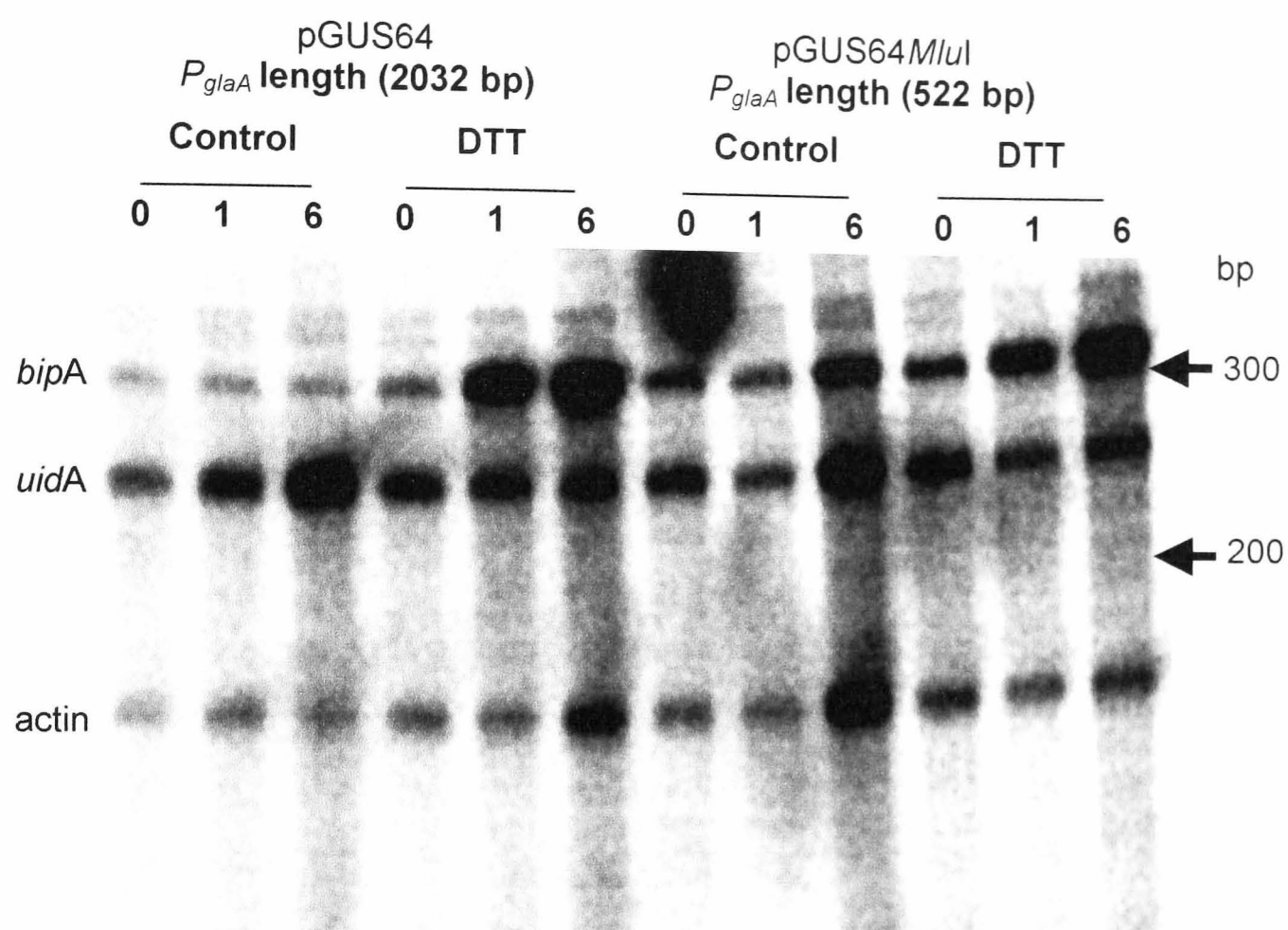


Figure 4.7

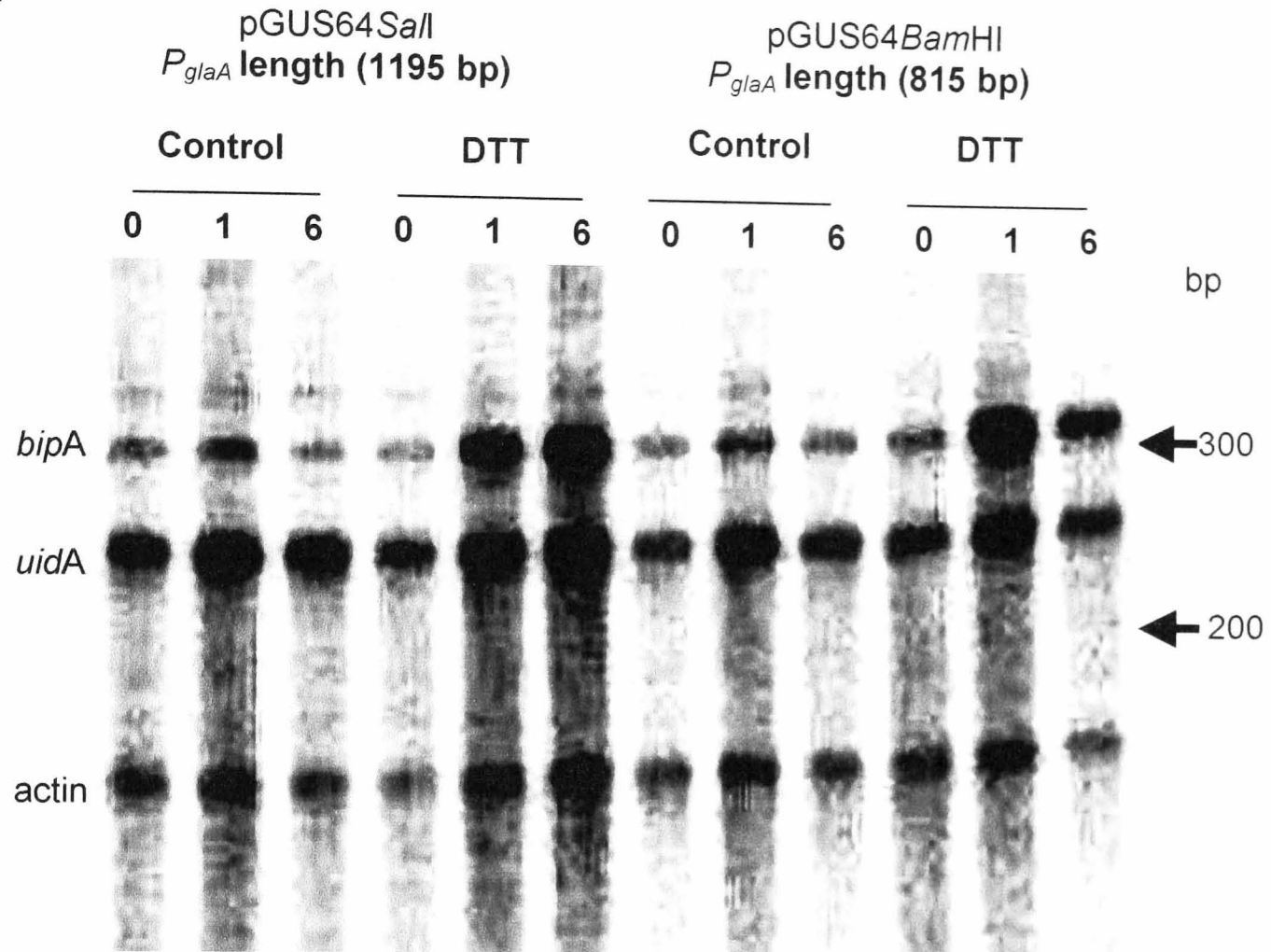
c.



Ribonuclease Protection Assay (RPA) showing the effect of DTT treatment on the transcription of *bipA*, *uidA* in *A. niger* GUS64 and GUS64Mlul strains. Actin was used to normalise loading of each RNA sample.

Figure 4.7

d.



Ribonuclease Protection Assay (RPA) showing the effect of DTT treatment on the transcription of *bipA*, *uidA* in *A. niger* pGUS64*SalI* and GUS64*BamHI* strains. Actin was used to normalise loading of each RNA sample.

4.3.5 DTT interferes with the fluorometric GUS assay

GUS activity from DTT treated cultures is significantly reduced compared to untreated controls. To test whether the residual DTT, that possibly remained after harvesting the mycelia, interferes with the GUS assay, DTT was directly added to the samples at a final concentration of 20 mM DTT. It was found that, after immediate addition of DTT to the supernatant, the GUS fluorescence activity was reduced about 60% compared to the control (Figure 4.8 a). Furthermore, incubation of the supernatant with the DTT at room temperature for 3 hours (Figure 4.8 b) or 6 hours (Figure 4.8 c) resulted in further lowering of the GUS activity by 80% and 85%, respectively, compared to the control. To further confirm and validate these findings, DTT at a final concentration of 20 mM was added to GUS and to a series of diluted 4-methylumbelliferone solutions (calibration standard). In both cases, a reduced level of the 4-methylumbelliferone fluorescence was detected. As Figure 4.8 (d and e) show, the level of GUS activity produced by the commercially-acquired GUS was reduced after DTT was added. To test whether the presence of DTT at a final concentration of 20 mM interferes with the fluorescent activity of the 4-methylumbelliferone the standard was prepared with and without DTT and a fluorimetric reading was taken immediately after DTT addition. As Figure 4.8 (f) shows, there was a reduction of the 4-methylumbelliferone fluorescence of about 26%.

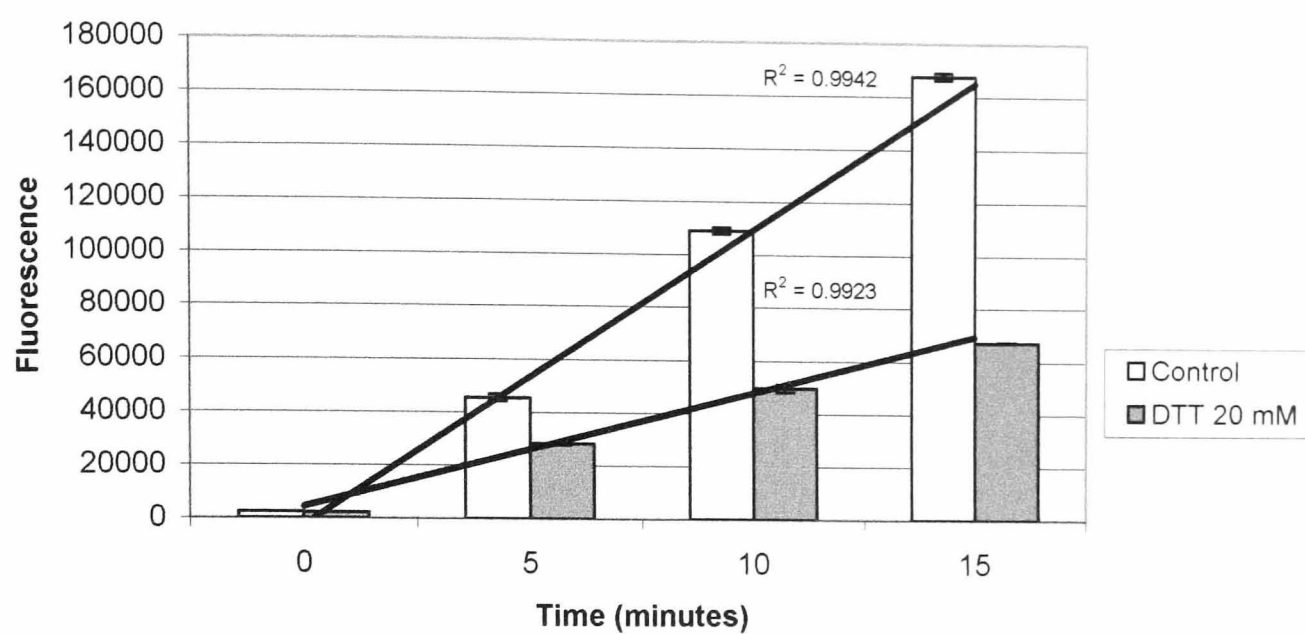
The conclusion from these studies is that DTT adversely affects the GUS assay but the required level of DTT in the GUS assay performed with extraction of *A. niger* is not known.

Figure 4.8 The effects of adding DTT (20 mM final concentration) to the supernatant of *A. niger* GUS64 reporter strain on the fluorescence activity of the product 4-methylumbelliferone. As a positive control, the GUS assay was performed using commercial GUS. To serve as a positive control for the fluorescence a solution of 4-methylumbelliferone was used. Fluorescence (arbitrary units) shown in these plots (a-f) represent the average \pm SEM of three triplicates.

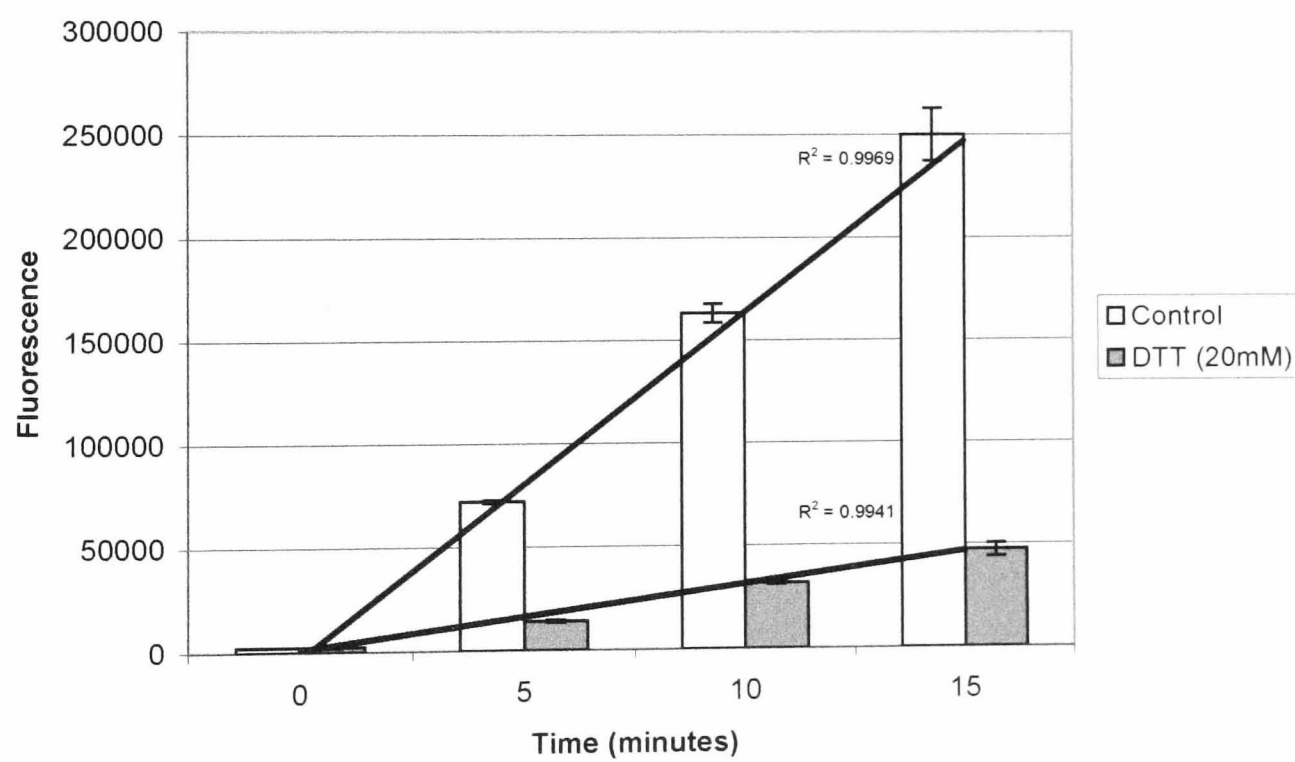
R^2 : for the trendline, a best fit line, is an indicator that ranges in value from 0 to 1 and reveals the linearity of the data set. A trendline is most reliable when its R^2 is at or near 1 (Zar, 1996).

- a. Gus activity in extract of *A. niger* GUS64. Assay carried out immediately after DTT addition at 20mM final concentration.
- b. Gus activity in extract of *A. niger* GUS64. Assay carried out 3hrs after DTT addition.
- c. Gus activity in extract of *A. niger* GUS64. Assay carried out 6hrs after DTT addition.
- d. Gus activity of GUS. Assay carried out immediately after DTT addition.
- e. Gus activity of GUS. Assay carried out 3hrs after DTT addition.
- f. Effect of adding DTT (20mM) on the fluorescence of the 4-methylumbelliferone usually used to prepare the standard curve.

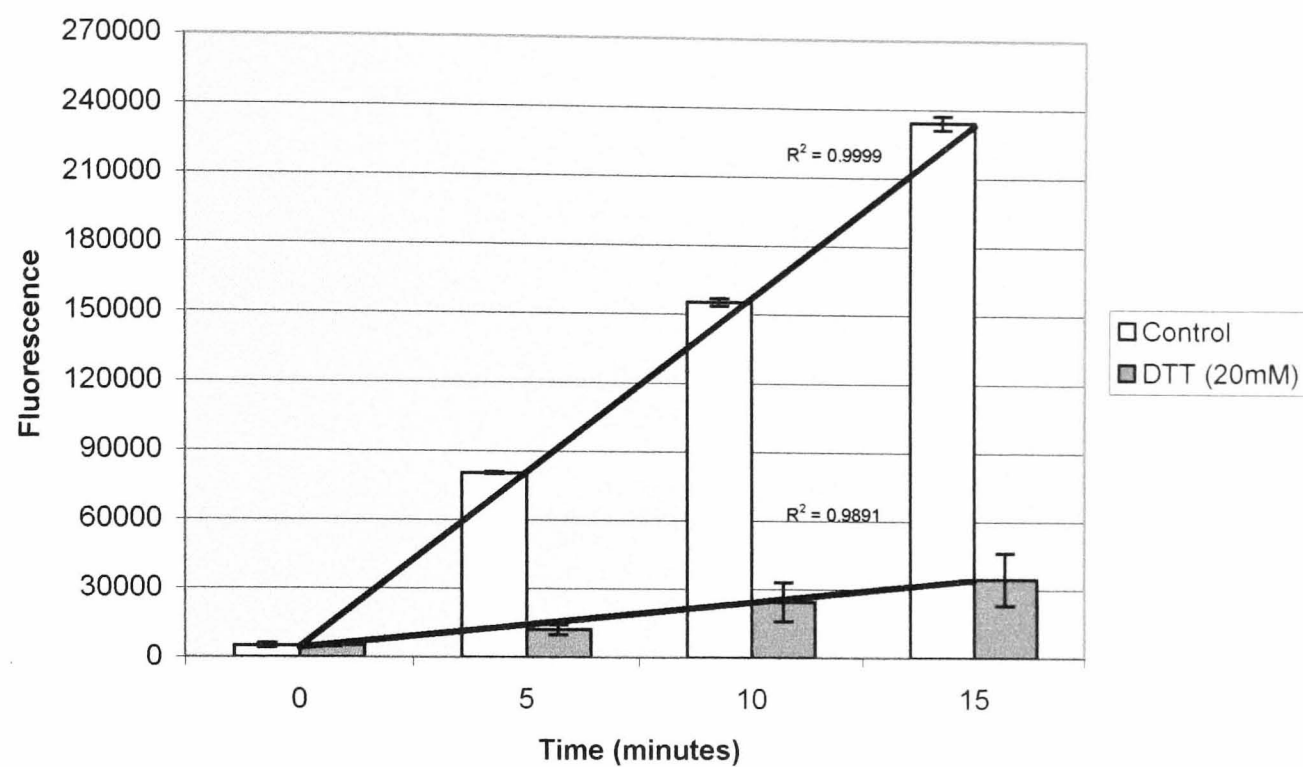
a.



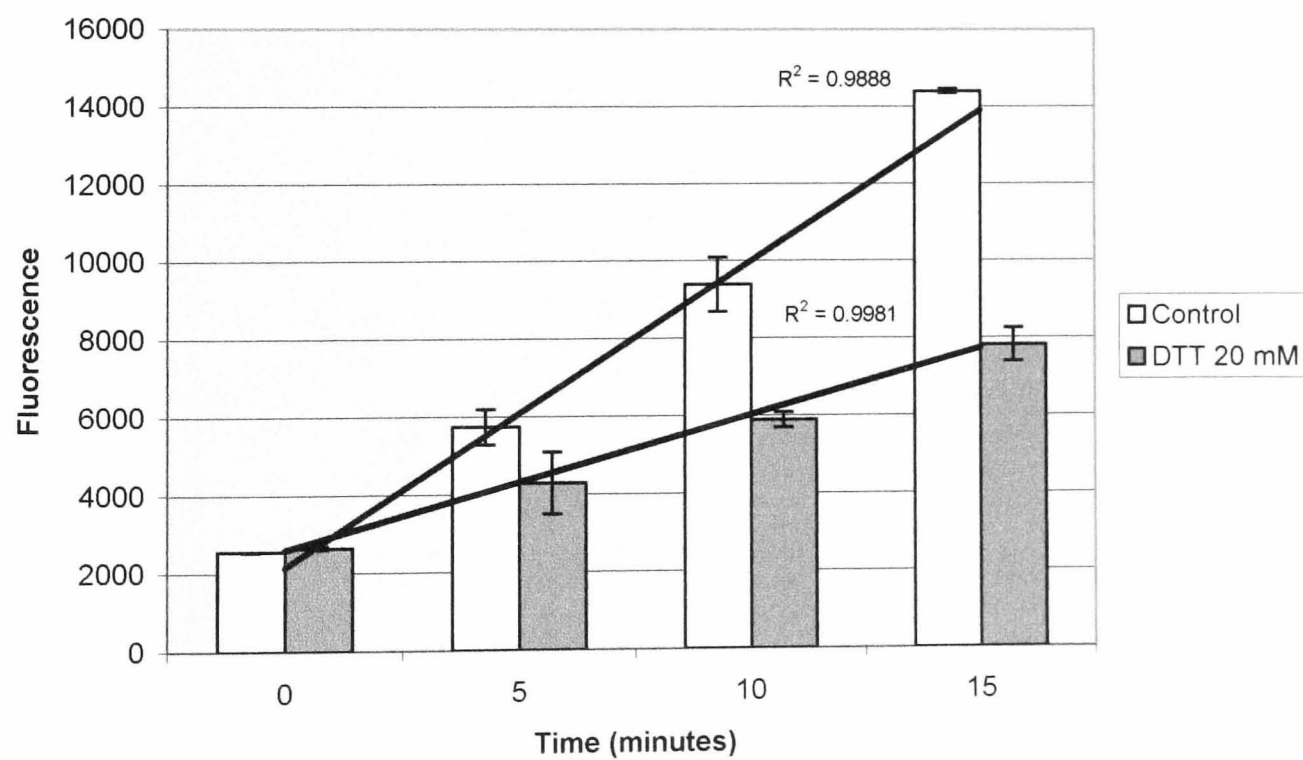
b.



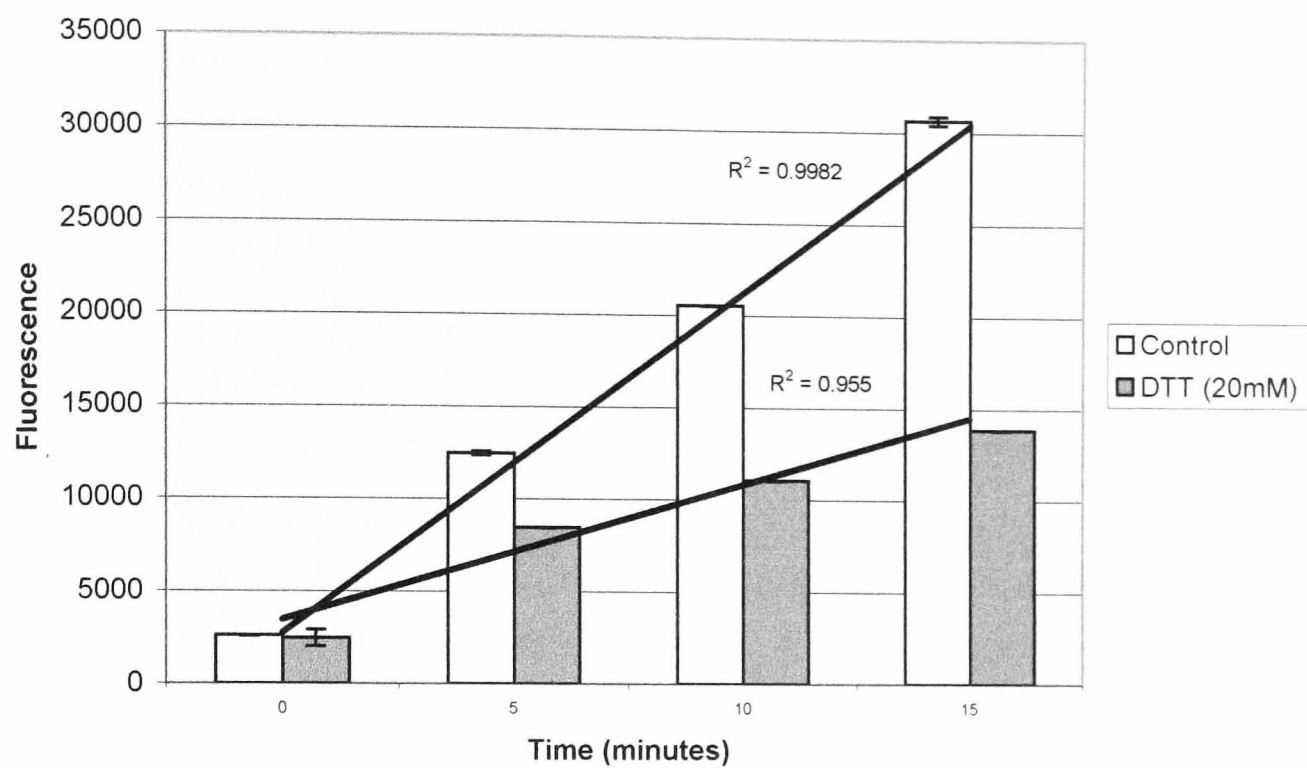
c.



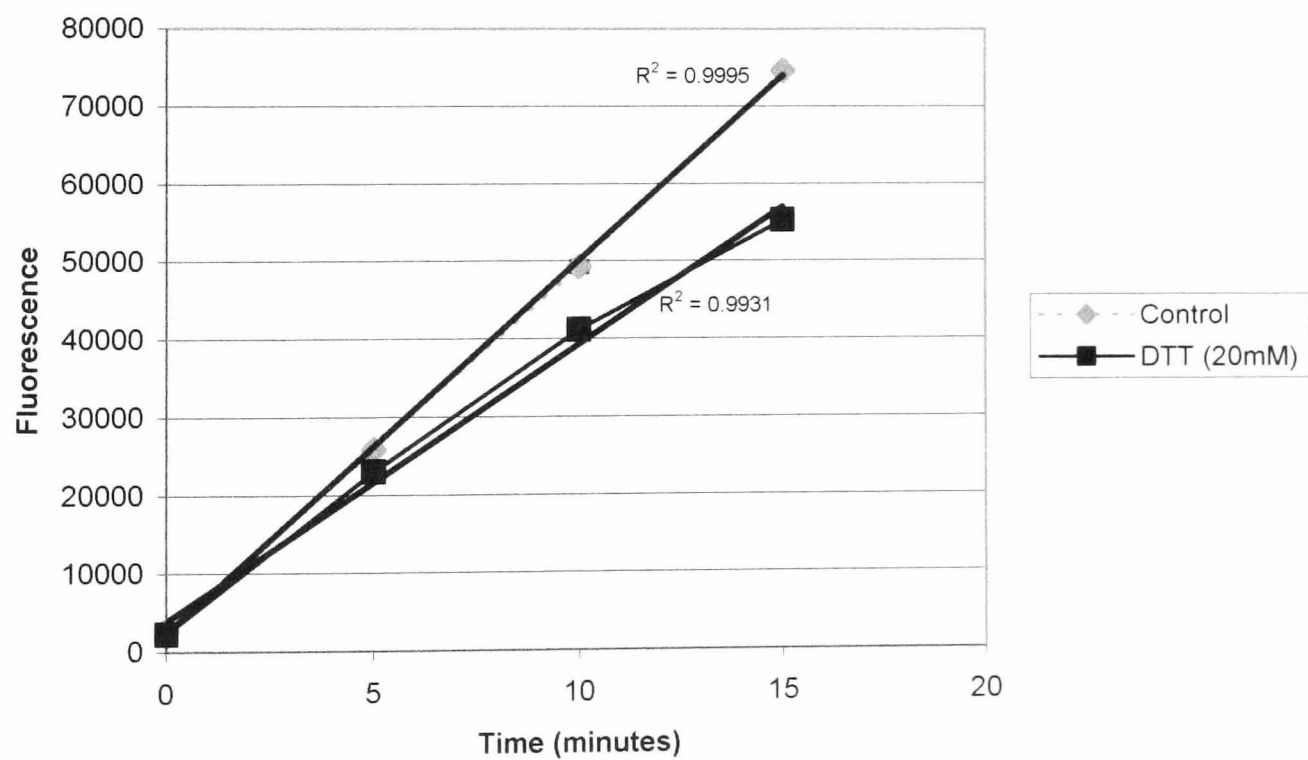
d.



e.



f.



4.3.6 Stability of the *uidA* transcripts in *A. niger* GUS64 and GUS64SaII strains is similar under different growth conditions

The stability of *uidA* mRNA was checked by RPA and RT-PCR, and at the protein level the GUS stability was checked by GUS assay. First, the stability of *uidA* transcripts was checked by RPA after switching from a medium with a P_{glaA} -inducing carbon source (starch) to a medium containing a non- P_{glaA} -inducing carbon source (xylose). Next, the size of *uidA* mRNA was checked partially by amplifying a fragment that was likely to contain the transcriptional start point(s) (Boel *et al.*, 1984a) (Figure 4.1) from *A. niger* reporter strains GUS64 and GUS64SaII that were treated with DTT. This was achieved by using a primer set in the RT-PCR experiments that amplify a DNA fragment from the putative transcriptional start site. Thus, these primers could amplify the target fragment if they could bind to their target site in the RT-PCR experiment, but if the transcriptional start site is in a different region or the mRNA was truncated, no fragment will be amplified with the expected size. However, the GUS level was not checked in mycelia that had been treated with DTT due to the observation that DTT may interfere with GUS assay (see section 4.3.5 in this Chapter).

4.3.6.1 Stability of *uidA* mRNA transcripts and GUS after switching to a non-inducing carbon source of P_{glaA}

The stability of *uidA* mRNA in *A. niger* strain GUS64 after switching the mycelia from ACMS/N/P to ACMX/N/P medium was checked by RPA (Figures 4.9 a, b and c). The half-life of the *uidA* mRNA is likely to be 20 minutes or less. However, it should be mentioned that, due to the significant drop in the *uidA* transcripts from the first time point (20 minutes), more time points between 0 and 20 minutes are needed for more accurate calculation of the declining rate of the transcripts.

The decreasing rate in the level of *uidA* transcripts calculated under non-inducing conditions (about 76% decrease after 360 minutes of medium-exchange) is comparable to the decreasing rate of the *uidA* transcript level seen under DTT treatment in the pGUS64 strain (72% decrease after 360 minutes of DTT

treatment (Figure 4.10)). These results suggest that, under DTT treatment, the induction of *uidA* expressed under the control of P_{glaA} is significantly reduced, resulting in net degradation of *uidA* transcripts somewhat similar to the degradation rate of *uidA* mRNA after changing to a medium with a represser of P_{glaA} (xylose) (Figure 4.10).

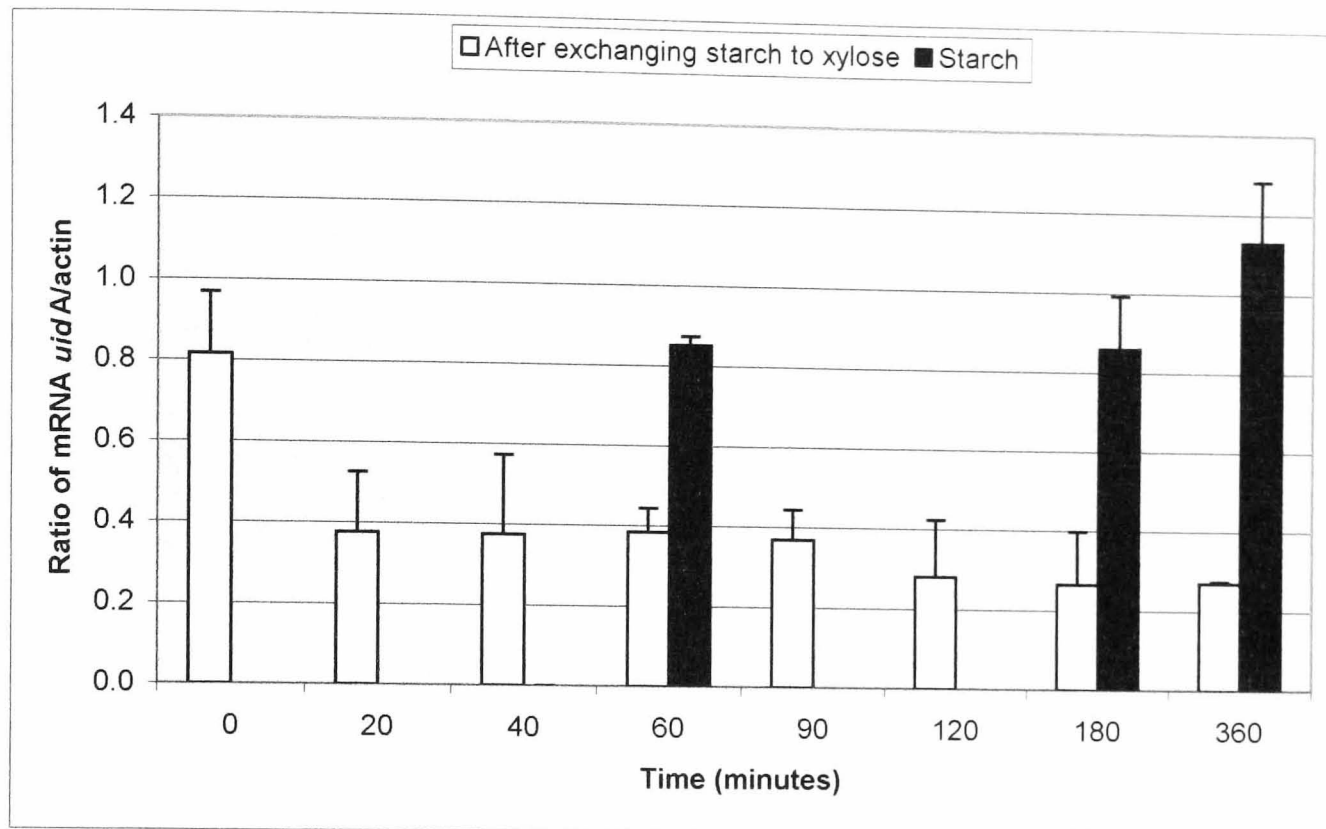
To further check the *uidA* reporter gene product at the protein level, GUS assays were carried out using the same samples used for carrying out the RPA in Figure 4.9a. The GUS enzyme levels were similar throughout the time course (Figure 4.9b). This indicates that there was no obvious degradation of the protein taking place during the time course, indicating a long half-life of the GUS protein. Also, by examining the control samples, where there is no medium-exchange, it can be seen that the protein level continued to increase. Correlating with this, the mRNA level for the same control samples continued to increase as can be seen in Figure 4.9(a).

Figure 4.9

- a. The effect of exchanging the medium from starch to xylose as a carbon source on the transcript level of *uidA* in *A. niger* GUS64 reporter strain.
- b. GUS-specific activity from *A. niger* GUS64 strain carrying the full length *P_{glaA}*. Sample from starch-xylose medium exchange.
- c. RPA for starch-xylose medium exchange experiment detecting the mRNA level of *bipA*, *uidA*, and actin in *A. niger* pGUS64 strain.

Exchanging the medium was carried out after 44 hours of growth in ACMS/N/P medium. The time point refers to the time of medium exchange. The S samples serve as a positive control (no medium exchange was done in those samples). The time points shown represent the time in minutes after the medium-exchange. Error bars are an average \pm SEM of three replicates.

a.



b.

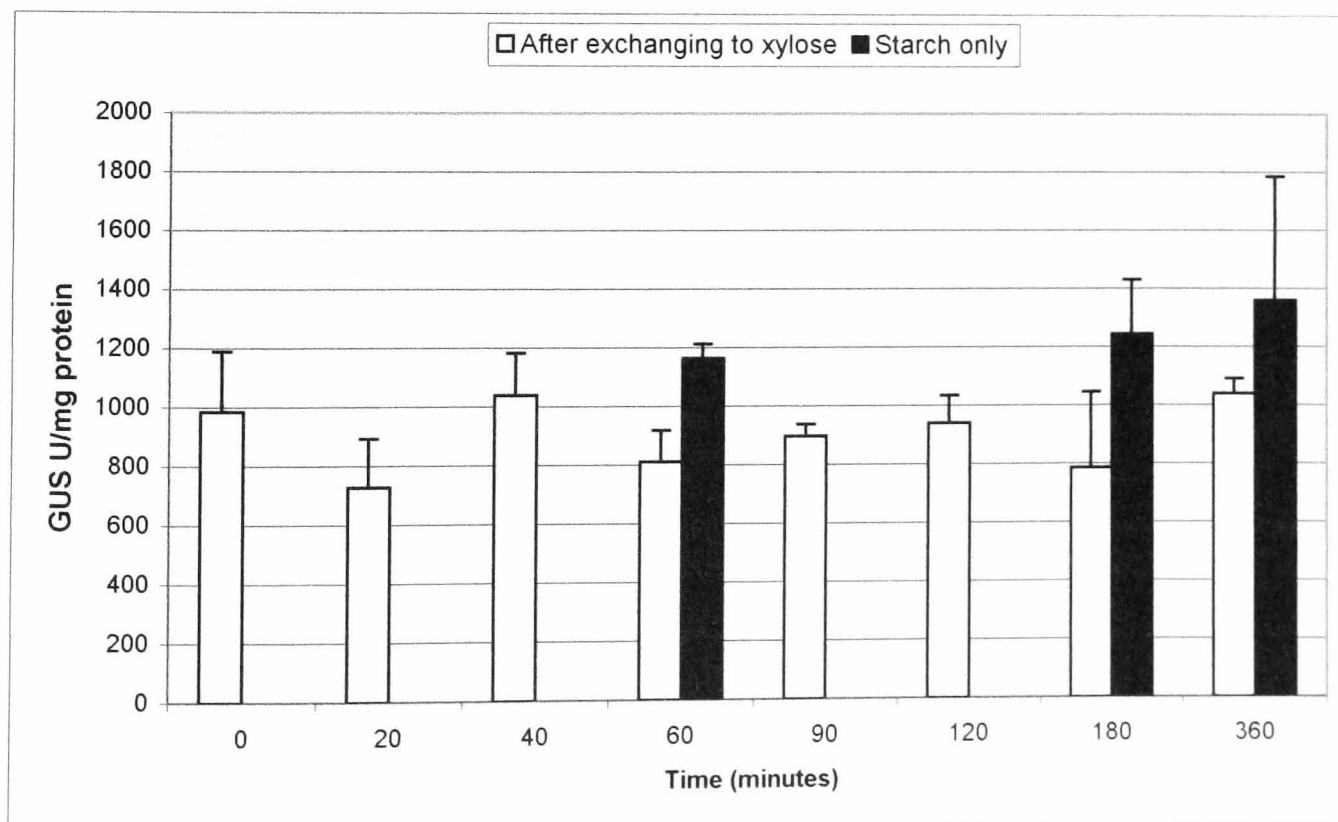


Figure 4.9
c.

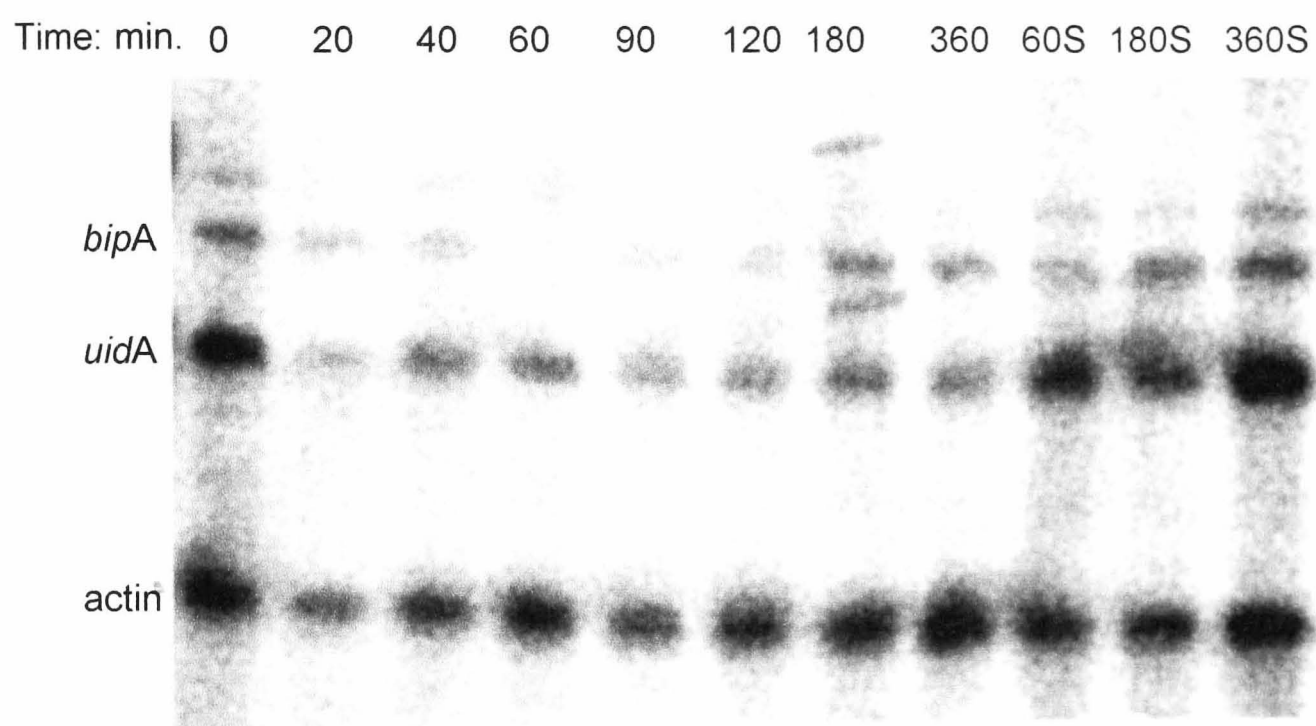
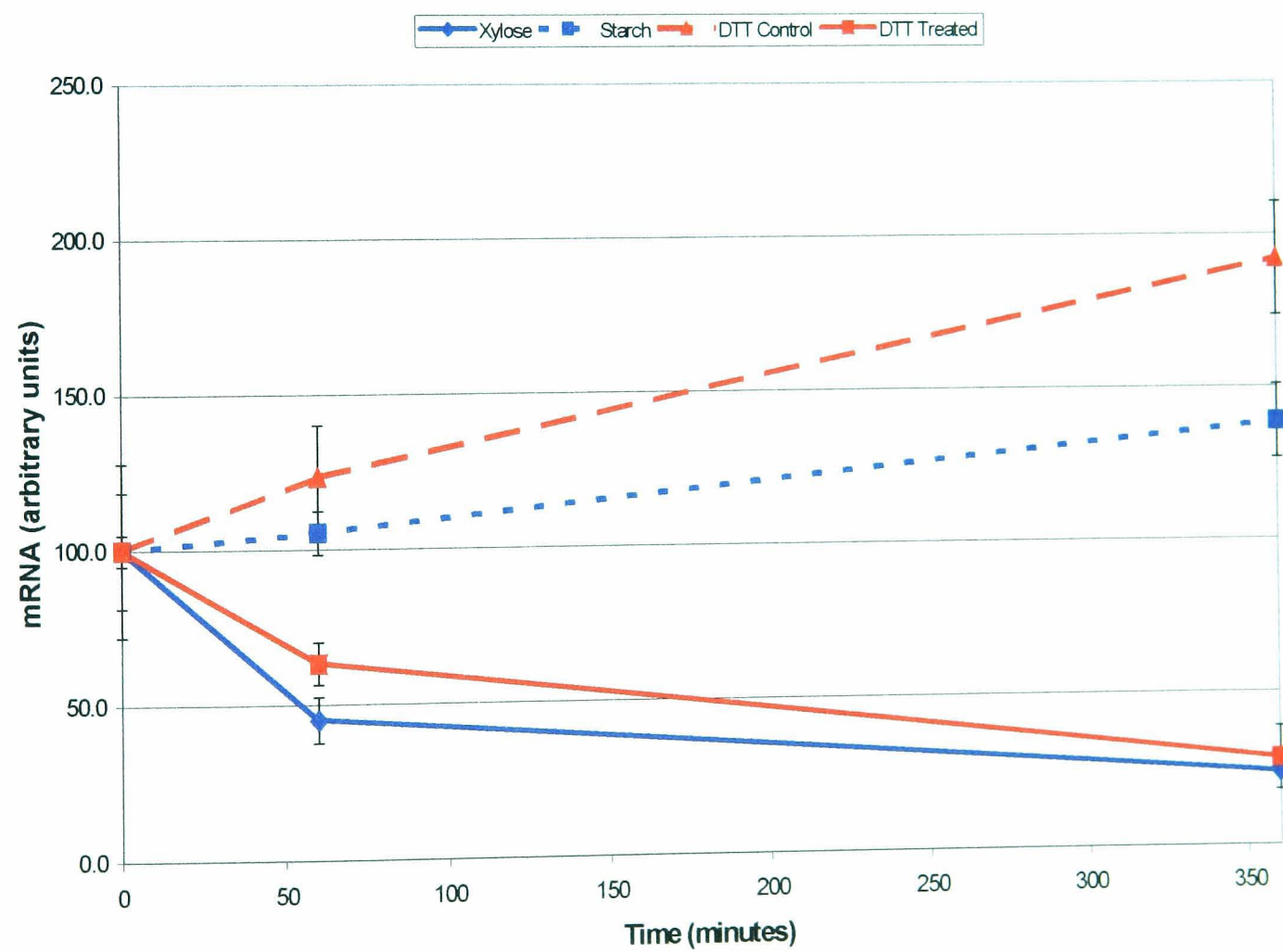


Figure 4.10 The expression levels of *uidA* mRNA under DTT treatment or after exchanging the medium to ACMX/N/P medium in *A. niger* GUS64 strain. The relative *uidA* expression is shown as the percentage of the *uidA* expression compared to that of 0 time point in the same experiment (100%).



4.3.6.2 Size of *uidA* cDNA under DTT treatment and medium exchange is similar in *A. niger* GUS64 and GUS64*SalI* strains

The approximate size of partially amplified *uidA* cDNA fragments from *A. niger* GUS64 and GUS64*SalI* reporter strains, under both DTT treatment and medium exchange, was determined by RT-PCR - as described in section 3.2.2 - and compared after electrophoresis through 2% agarose gels. The aim of these experiments was to determine whether DTT treatment in *A. niger* GUS64 and GUS64*SalI* reporter strains, that show different *uidA* mRNA declining patterns, under DTT treatment would change the stability of *uidA* mRNA by either changing the transcriptional start point or by truncating the *uidA* mRNA. In addition, if DTT treatment results in changing the transcriptional start point or shortening the translated *uidA* mRNA are these changes similar in both reporter strains, GUS64 and GUS64*SalI*? Two different combinations of primer sets and total RNA were used to determine the approximate size of two different *uidA* cDNA fragments. The reason for amplifying two different fragments separately instead of amplifying them as one fragment is that the transcriptional start point is not definite. More specifically, Boel *et al.* (1984) reported, based on the cDNA synthesized, that 70% of the glucoamylase mRNA species have a 5'-untranslated region upstream of the translational start codon of 44 nt, while 30% of the transcripts would have a 5' end of 69 nt. The putative transcriptional start point (69 nt) was checked using primer set II which contains P2F primer that covers it. Thus, if these primers could amplify the target fragments this would mean that no truncation has taken a place at least in the site for these primers, but if the transcriptional start site is in a different region or the mRNA was truncated, no fragment will be amplified with the expected size.

The first fragment (fragment I) that was amplified using set I primers (as described in section 4.2.2), should result in the amplification of a 621 bp fragment corresponding to coordinates +1, first nucleotide in the translation start codon ATG, to +621 in the *E. coli uidA* reporter gene if the *uidA* transcripts have not been

shortened due to the DTT treatment or medium-exchange. RT-PCR for fragment I under medium-exchange (from ACMS/N/P to ACMX/N/P) or DTT treatment showed similar band sizes compared to their control bands (Figures 4.11 and 4.12) in both *A. niger* reporter strains GUS64 and GUS64*SalI* reporter strains. These results suggest that there is no truncation in the first 621 bp of the *uidA* gene. Amplifying fragment I from medium-exchange samples would serve as a positive control for the effect of DTT on the stability of the *uidA* mRNA. More specifically, if truncation occurs only in the cDNA of fragment I prepared using the RNA samples from DTT treated cultures, but not in the one obtained from medium-exchange samples, that might suggest that any mRNA truncation is due to the presence of DTT itself. Also, due to the high sensitivity of the RT-PCR system, the number of PCR cycles for these experiments was reduced to 25 cycles to avoid amplifying a very low target to the saturation level. Further analysis was conducted using nuclear run-on as described in the next Chapter.

The other fragment (fragment II) prepared using set II primers (as described in section 4.2.2). was a 365 bp fragment corresponding to coordinates –81 to +284 in the *A. niger* *P_{glaA}* and the *E. coli uidA* reporter gene, respectively. Successful amplification of fragment II using the P2F primer which covers the possible transcriptional start point might confirm not only the previously reported transcriptional start point (Boel *et al.*, 1984a), but also that no changing of the transcriptional start point had taken place after 1 hour of DTT treatment of GUS64 and GUS64*SalI* reporter strains (Figure 4.13). All the above results suggest that the decline in *uidA* mRNA under DTT treatment is not likely to be due to mRNA stability. Rather, it probably takes place at the transcriptional level in both *A. niger* reporter strains GUS64 and GUS64*SalI*. To confirm whether the decline in *uidA* mRNA under DTT treatment is occurring at the transcriptional level, nuclear run-on studies were carried out (Chapter 5).

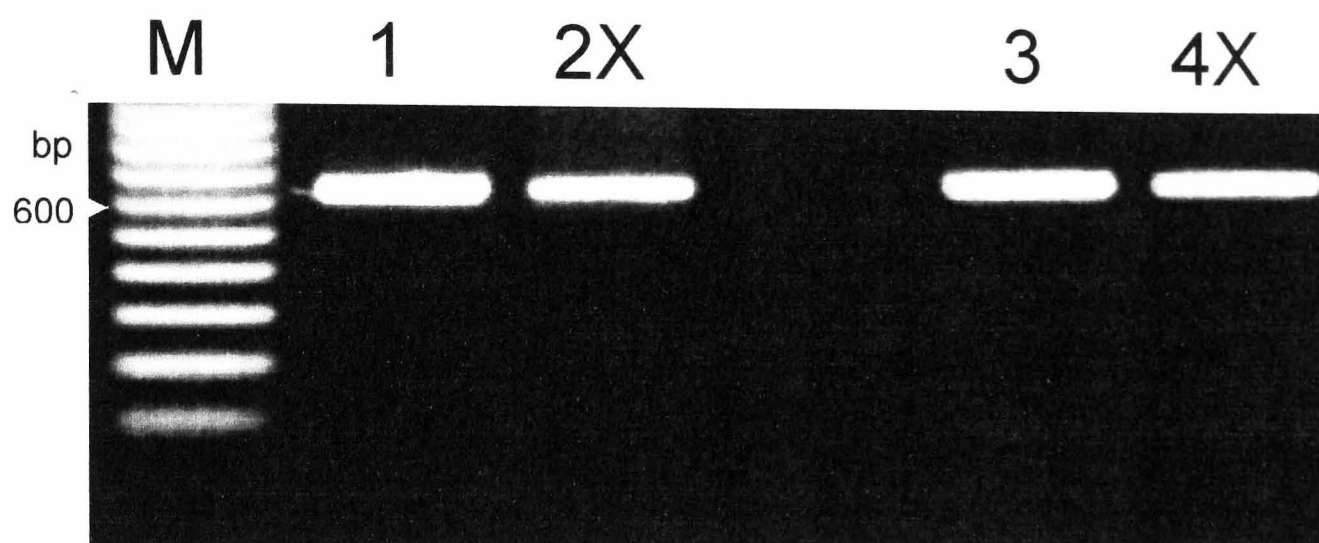


Figure 4.11 RT-PCR fragment I corresponds to coordinates +1 to +621 in the *uidA* reporter gene. Total RNA was used as a template for the RT-PCR from *A. niger* cultures grown in ACMS/N/P or after 6 hours of switching to ACMX/N/P medium (X).

Lanes:

M: 100 bp DNA ladder (Invitrogen, UK)

1 *A. niger* GUS64 strain grown in ACMS/N/P (no medium-exchange)

2X *A. niger* GUS64 strain after 6 hours of exchanging the medium to ACMX/N/P

3 *A. niger* GUS 64sall strain grown in ACMS/N/P (no medium-exchange)

4X *A. niger* GUS 64sall strain after 6 hours of exchanging the medium to ACMX/N/P

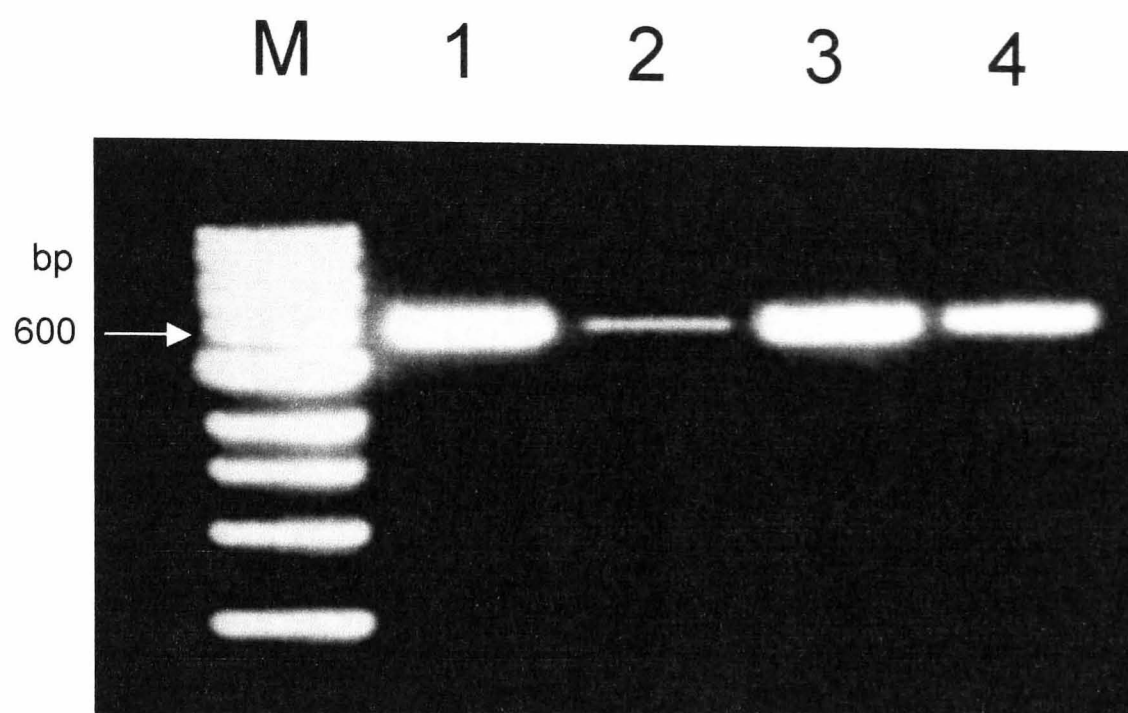


Figure 4.12 RT-PCR of fragment I corresponds to coordinates +1 to +621 in the *uidA* reporter gene. Total RNA was used as a template for the RT-PCR from *A. niger* cultures grown in ACMS/N/P and treated with equal volumes of either water or 20 mM DTT for 6 hours.

Lanes:

M: 100 bp DNA ladder (New England BioLabs, UK)

1 *A. niger* GUS64 strain control (water treated)

2 *A. niger* GUS64 strain after 6 hours of DTT addition

3 *A. niger* GUS 64*saII* strain control (water treated)

4 *A. niger* GUS 64*saII* strain after after 6 hours of DTT addition

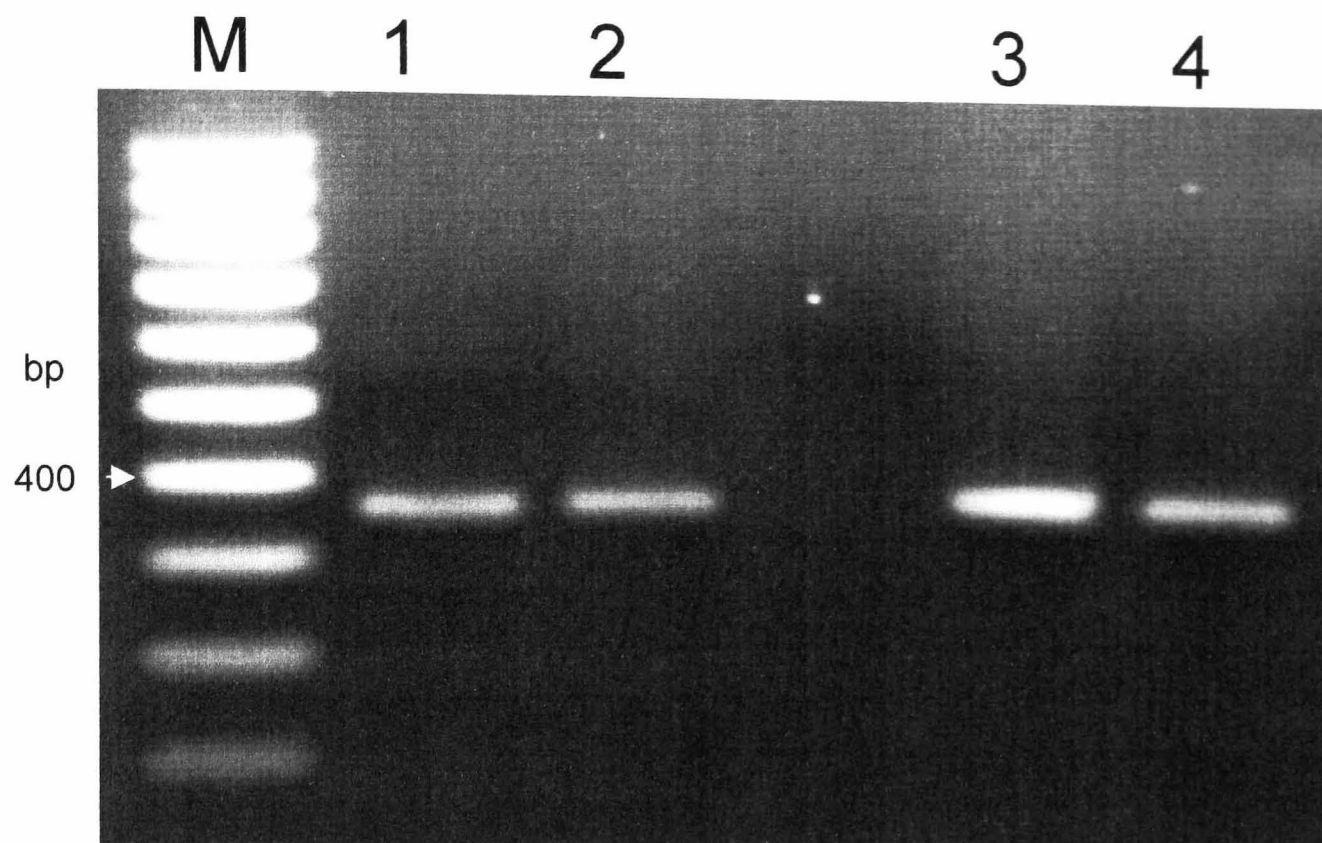


Figure 4.13 RT-PCR of fragment II, a 365 bp fragment, corresponds to coordinates –81 to +284 in the *A. niger* *P_{glaA}* and the *E. coli uidA* reporter gene, respectively. Total RNA was used as a template for the RT-PCR from *A. niger* cultures grown in ACMS/N/P and treated with equal volumes of either water or 20 mM DTT for 1 hour.

Lanes:

M: 100 bp DNA ladder (Invitrogen, UK)

1 *A. niger* GUS64 strain control (water treated)

2 *A. niger* GUS64 strain after 1 hour of DTT addition

3 *A. niger* GUS 64*SalI* strain control (water treated)

4 *A. niger* GUS 64*SalI* strain after 1 hour of DTT addition

4.4 Discussion

The P_{glaA} of *A. niger glaA* is frequently used for overexpression of native and heterologous genes in filamentous fungi (Table 1.4). The full sequence of 2.032 kb of P_{glaA} is shown in Figure 4.4. Several putative regulation sites present within the P_{glaA} can be seen in Figure 4.1, most of which have been reported previously (Verdoes *et al.*, 1994). The reporter gene (*uidA*), controlled by the truncated P_{glaA} , was up-regulated by starch and down-regulated by glucose or xylose. Although regulation of *A. niger P_{glaA}* in different carbon sources has been well-characterized in several studies (Fowler *et al.*, 1990; Verdoes *et al.*, 1994), results of these studies do not always completely correspond with each other. For instance, Fowler *et al.* (1990) reported that xylose had no effect on *glaA* expression levels when mixed with maltose or glucose-grown cultures of *A. niger*, whereas Verdoes *et al.* (1994) reported that the expression level of the reporter gene controlled by P_{glaA} when xylose was mixed with either maltose or glucose was intermediate to the *glaA* expression level when it was expressed in maltose or glucose only. More specifically, in the study of Fowler *et al.*, (1990) *glaA* repression by xylose was overcome by the addition of maltose or glucose to the levels seen when maltose or glucose are used as the sole carbon source, whereas the study by Verdoes *et al.* (1994) showed that maltose or glucose cannot completely overcome the xylose repression. Verdoes *et al.* (1994) suggested that the reason for these different observations was the fact that strains and growth conditions were not completely comparable in both studies.

Another observation on *glaA* regulation described by Fowler *et al.* (1990) is that, when xylose is mixed with starch, it can prevent the induction of glucoamylase by starch. This observation may indicate that starch itself is not the signal for *glaA* synthesis. The suggestion that starch itself is not the signal for initiating *glaA* synthesis might be supported by analogy with the observation made in the case of cellulose. It was shown in *T. reesei* that cellulose itself is not the inducer of cellobiohydrolase. Instead, a basal level of constitutive cellulase might

degrade cellulose to smaller saccharides that can be taken up by the cell and converted into inducers that can initiate the induction (Suto and Tomita, 2001).

The specific activity of GUS produced by each of those strains in my study cannot be directly compared with those data reported by Verdoes *et al.*, (1994) since the carbon sources were not identical (i.e. in this study starch was used, whereas in Verdoes *et al.*, (1994) maltose was used), as well as some other growth conditions (e.g. inoculum size, growth period, and carbon source concentration). However, the general pattern of GUS expression under the control of P_{glaA} agreed with what is generally known about the regulation of *glaA* in different carbon sources. One interesting observation made in the *A. niger* reporter strains carrying the truncated *glaA* promoters is that hardly any GUS production was detected, except for the strain carrying 2032 bp of P_{glaA} , when these strains were grown in glucose. This may suggest that a region(s) which is required for a high level of expression when grown in glucose is located in a region more than -1.195 kb upstream of the translational start, since this promoter region is only present in the GUS64 reporter strain. This could be supported by the following observations. Firstly, the CCAAT motif, which has been considered a functional element essential for high-level expression for many genes in filamentous fungi (Brakhage *et al.*, 1999), is present twice in the P_{glaA} . One of these two CCAAT sequences is only present in the GUS64 reporter strain, and is located -1998 to -1994 bp upstream of the translational start (Figure 4.1). In a different *A. niger* strain (T21) two CCAAT sequences within P_{glaA} have been reported to act as a binding site for a positive transcriptional factor in *A. niger* (Qiu *et al.*, 2002). More specifically, DNase I footprinting analysis showed that a protein, named AngCP extracted from starch-induced mycelia, binds to two regions containing CCAAT sequences in P_{glaA} located -374 to -344 and -484 to -414 bp relative to the *glaA* translational start codon. Moreover, it has been shown that the introduction of 8 copies of an 84 bp fragment containing the CCAAT motif into P_{glaA} , over a region extending from about 0.4 – to – 1.1 kb of P_{glaA} of *A. niger* T21 strain, increased the expression of the target gene about 20 fold compared to the presence of a single copy of that fragment (Liu *et al.*, 2003). Moreover,

mutational experiments have shown that a CCAAT motif, present within a 36 bp fragment (-327 - to - 292 bp upstream of the translational codon) in the *amyB* promoter, is involved in high-level expression of the *amyB* gene, but not in maltose induction (Kato *et al.*, 1997). So the observation that the CCAAT motif plays an important role in high level expression of P_{glaA} , as shown by the above studies and others (Brakhage *et al.*, 1999), may suggest that deletion of one CCAAT sequence out of the two CCAAT sequences present in the P_{glaA} in *A. niger* reporter strains used in this study might result in 'weaker' promoters. When *A. niger* was grown in glucose no detection of GUS was possible in reporter strains where one CCAAT sequence was deleted. Furthermore, a similar observation has been reported in glucose-grown cultures of a different strain of *A. niger*, where expression of glucoamylase was driven by a truncated region of the P_{glaA} . More specifically, a region between ~0.5 and ~1.9 kb upstream of the translational start codon was removed (Fowler *et al.*, 1990). This removed region contains two putative CCAAT sequences out of a total of three putative CCAAT sequences present in the P_{glaA} of the *A. niger* strain used in that study, which is a different *A. niger* strain to those used in this study. Although these deletion studies were complicated by the use of multicopy transformants with their construct integrated at various genomic loci, it was observed that the level of expression was significantly low only when the transformant strain (mentioned above) was grown in glucose. Furthermore, the regulation of P_{glaA} in different carbon sources was studied by MacKenzie *et al.* (1994). Data reported in that study agree with the observations made in this study. More specifically, MacKenzie *et al.* (1994) reported that the yields of endogenous glucoamylase could be ordered with respect to growth on the following carbon sources: soluble starch > maltose > glucose > xylose (MacKenzie *et al.*, 1994), further confirming that glucose does not completely repress P_{glaA} .

Since the *A. niger* reporter strains used in this study carry a single copy of the reporter, a more sensitive technique than northern blotting was desirable for studying the expression of these reporter genes under ER stress. RPA has many advantages over northern blots. RPA can detect a very low level of target RNA (i.e. as little as 5 femtograms), RPA is more tolerant of partially-degraded RNA than

northerns and, more importantly, multi-probe assays are much easier to perform with RPA. The positive and negative controls (Figure 4.6) confirm the validity of the system used to perform the study.

RPA analysis at this stage suggested that the decline of the *uidA* mRNA under ER stress triggered by DTT treatment was mediated through a region of P_{glaA} between 1.195 and 2.032 kb upstream of the translational start point. Results up to this stage suggest, indirectly, that the decline in transcript level of the reporter gene under the control of P_{glaA} is occurring at the transcriptional level. These results include the similarity between the declining rates of *glaA* mRNA when exposed to DTT and those calculated in the absence of *de novo* synthesis of *glaA* mRNA as studied by medium exchange experiments (Al-Sheikh *et al.*, 2004), and discussed in Chapter 3. Also, a similar scenario is seen with the *uidA* reporter gene under the control of P_{glaA} (Figure 4.10). Recently, the apparent transcriptional down-regulation of genes encoding secreted proteins has also been observed in *T. reesei* (Pakula *et al.*, 2003) where it was termed RESS (Repression Under Secretion Stress). In their study, deletion analysis of the *cbh1* promoter using a reporter gene system was used. The reporter gene (*lacZ* gene of *E. coli*) was expressed in *T. reesei* exposed to DTT for a time-course of 6 hours under either a full-length 2.2 kb *cbh1* promoter or a shortened promoter of 0.161 kb. The shortened promoter contains a putative TATA-box and the transcriptional start site and was shown to be functional. Northern blot analysis showed that the reporter gene was subjected to down-regulation only when expressed under the control of 2.2 kb *cbh1* promoter. However, the results of Pakula *et al.* (2003) and the results from my study up to this stage do not establish beyond doubt that the down-regulation is at the transcriptional level. In order to establish the transcriptional down-regulation definitively, the *de novo* mRNA level of the gene in the study should be monitored (Chapter 5).

Several lines of study were consistent with the transcriptional down-regulation of *uidA* under secretion stress being transcriptional rather than a function of mRNA stability. Firstly, the *uidA* mRNA stability was studied under the condition of changing the carbon source in the medium from an inducing to a non-

inducing carbon source. The stability of *uidA* mRNA was then studied (using RPA) to determine if the decline in mRNA level was similar in repressed conditions as well as under DTT treatment. Secondly, since the steady-state mRNA levels of *uidA* detected in the reporter strains shown in Figure 4.7 did not show an obvious increase during the time-course period used (6 hours), except for the GUS64 reporter strain, the growth conditions were optimised to improve the induction of the *uidA* gene to a level where an increase in the *uidA* transcript level was observed during the DTT time-course. This was achieved by using a medium-exchange. The final step was to detect the *de novo* mRNA synthesis driven by the 2.032 and 1.195 kb *P_{glaA}* under DTT conditions. The last two steps will be discussed in Chapter 5.

Attempts to detect the effect of transcriptional down-regulation of the *uidA* gene at the protein level were unsuccessful. The addition of DTT at a final concentration of 20mM in the reaction buffer of the GUS assay interferes with detecting GUS activity in the assay. The observation that the incubation of DTT with the supernatant samples for 3 or 6 hours at room temperature resulted in further lowering of the GUS activity by about 20% may also indirectly suggest the possibility of degradation of GUS protein by extracellular proteases. Although PMSF, a serine protease inhibitor, was added to the mycelial extract (Chapter 2), it may not completely prevent GUS degradation. It has been reported previously that PMSF partially restricted the degradation of a heterologous protein, porcine pancreatic phospholipase A₂ (PLA₂), in *A. niger* cultures (Archer *et al.*, 1992), but different proteins are susceptible to degradation by different proteases.

The addition of DTT to the calibration standard resulted in a reduction in the fluorescence activity of 4-methylumbelliferone, but not as severe as the addition of DTT to the reaction buffer. This may indirectly suggest that DTT interferes mainly with the GUS assay.

At low concentrations, DTT stabilizes enzymes and other proteins which possess free sulfhydryl groups, and has been shown to restore activity lost by oxidation of these groups *in vitro*. DTT is also widely used for protein denaturation in SDS-PAGE electrophoresis. Although β -mercaptoethanol, another reducing

agent, was a component of the extraction buffer at a final concentration of 5mM, it seems that the presence of 20mM DTT in the GUS assay results in a reduction of GUS activity. However, these data do not explain why or how DTT interferes with GUS assay. No further investigation of this observation was carried out since it is not directly related to the main objective of this study.

The initial study of *uidA* mRNA stability suggested *uidA* transcripts in *A. niger* reporter strains, GUS64, GUS64*SalI*, GUS64*BamHI* and GUS64*Miul*, treated with DTT (Figure 4.7), were synthesised during the 6 hours time-course, and were not carried over from 0 time point. This is supported by the observation in Figure 4.9a, which showed that the half-life of *uidA* mRNA is likely to be ca. 20 minutes. Several other conclusions can be drawn from results shown in Figure 4.9. Firstly, these results confirmed that the reporter gene under P_{glaA} is regulated at the transcriptional level. Secondly, the reporter protein GUS is stable after the medium exchange (to non inducing carbon source) and is likely to have a half-life longer than 6 hours (length of time-course used in this experiment). Finally, these results showed that, under inducing conditions, the increase in the mRNA level correlates roughly with the protein level, but the protein has a long half-life as it is not degraded under non-inducing conditions.

The RT-PCR analysis to check the approximate size of partially-amplified *uidA* cDNA fragments from *A. niger* suggests that mRNA truncation is unlikely to take place under DTT treatment or medium-exchange at least within the 621 bp of the amplified fragment. Truncation of mRNA has previously been reported with expression of the heterologous gene encoding α -galactosidase in *A. niger* (Gouka *et al.*, 1997a; Gouka *et al.*, 1997b). Also, the putative transcriptional start point, located -69 nt upstream of the translational start point, was likely to be confirmed using a primer set that covers it. Furthermore, the similarity in size of the fragments covering the transcriptional start point, suggests that it was the same under DTT treatment and medium-exchange. If the transcriptional start point is located further upstream (i.e. upstream of the putative one), a different primer set could be necessary.

5 Transcriptional down-regulation of *glaA* under secretion stress at the nuclear level

5.1 Introduction

The results presented in the previous chapters show that under the conditions of ER stress there was a selective down-regulation of the gene encoding glucoamylase, but not the gene encoding non-secreted protein actin. Decreased mRNA stability is a common control mechanism of protein expression in various organisms (Anderson and Parker, 1998; Gouka *et al.*, 1997a; Guhaniyogi and Brewer, 2001; Vilela and McCarthy, 2003). One studied example of this mechanism is regulation of *S. cerevisiae* genes subject to glucose repression (Prieto *et al.*, 2000). In order to eliminate the possibility of *uidA* mRNA stability being responsible for the transcription down-regulation effects and also to confirm that the effect is truly transcriptional, the *de novo* mRNA synthesis should be examined under ER-stress conditions triggered by DTT. This can be done using nuclear run-on transcription assays. Nuclear run-on assays are transcription assays designed to examine the genes being transcribed in a cell nucleus at a specific time (Farrell 1997). The principle involved is that intact nuclei are isolated and these contain transcription complexes stalled on the DNA template due to acute loss of ribonucleotide substrates. The transcription that has been halted at the time of isolating the nuclei can be continued again *in vitro* after the addition of new ribonucleotides. Thus, the genes being transcribed under the specific conditions can be studied. By comparing the amount of gene-specific radiolabelled mRNA synthesized in nuclei that have been isolated from samples subjected to two different conditions (i.e. control and treated), the transcriptional activities of target genes can be studied (Figure 5.1). Thus, the nuclear run-on assay is useful in assessing whether changes in mRNA levels are a function of transcription or of subsequent RNA degradation or transport. The critical step that should determine the assay quality is the isolation of intact nuclei.

Nuclear Run-on Transcription

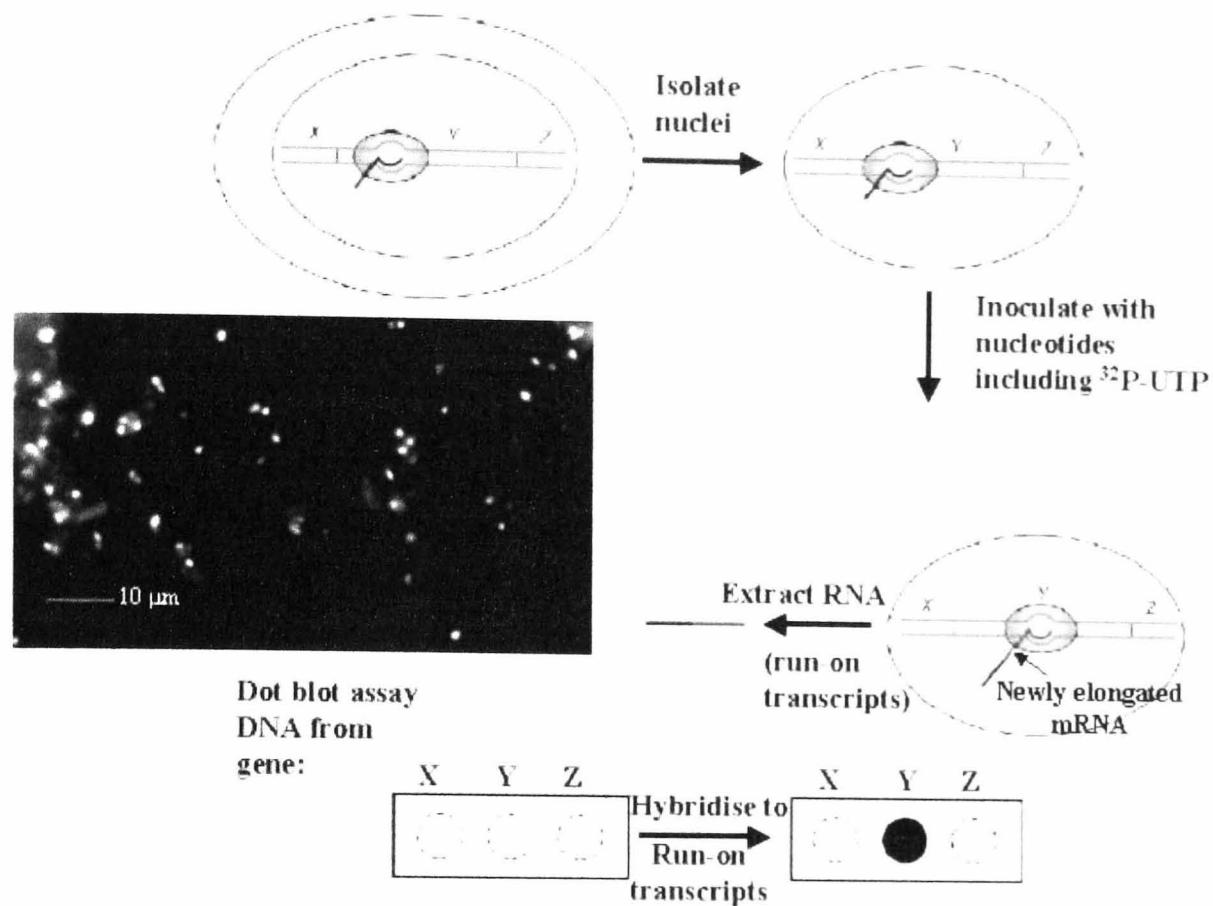


Figure 5.1 Nuclear run-on transcription assay. Nuclei are first isolated from mycelia and incubated with ^{32}P -labelled UTP and unlabelled NTPs to label nascent RNA transcripts. ^{32}P -labelled RNA is purified and used to detect specific RNA transcripts by hybridisation to DNAs immobilized on a nylon membrane. The newly synthesised mRNA will hybridise to its target. The isolated nuclei were checked under the fluorescence microscope after staining with ethidium bromide (shown in the photo).

Nuclear isolation must be carried out in such a way to preserve RNA polymerase activity and nuclear structure (Marzluff *et al.*, 1973; Marzluff *et al.*, 1974). In spite of this critical step, the sensitivity of the assay is high for the following reasons. The labelled RNA is single-stranded and the renaturation step that occurs with use of double-stranded probes, which may reduce the effective concentration of the probe in the hybridisation solution, is avoided. Also, the activity is highly specific, since the RNA is labelled continuously along its length compared with the use of end-labelled probes (Farrell 1997). In order to facilitate the detection of the transcripts of a gene that is present as a single copy, the nuclei should be isolated under optimal growth conditions for transcription induction for that gene. In this Chapter, the growth conditions have been improved to allow high levels of *uidA* induction from *A. niger* reporter strains. That was accomplished by carrying out medium-exchange. After improving the growth conditions for P_{glaA} induction, RPA and nuclear run-on experiments were performed to further study the transcriptional down-regulation phenomenon. Also, the effect of DTT treatment on the viability of *A. niger* cultures was determined.

5.2 Materials and Methods

5.2.1 Media-exchange and growth conditions

A. niger reporter strains were grown in ACMX/N/P for 44 hours, as described in Chapter 2 section 2.3, then mycelia were washed with ACM medium with no carbon source and transferred immediately to ACMS/N/P medium where the pH was adjusted with HCl to the pH of the medium to be exchanged (ACMX/N/P) (pH 3.5-4.0) (Figure 3.6) and at similar temperature, 28°C. Mycelia were harvested at the time mentioned in each experiment, as described in Chapter 2 section 2.4.

5.2.2 Nuclear run-on Assay

The nuclear run-on assay is composed of four main steps. These steps are described below, and include isolation of the nuclei, nuclear run-on transcription assay, hybridisation and washing of the DNA dot blot.

5.2.2.1 Isolation of the nuclei

Nuclei were isolated using the method described by Schuren *et al.* (1993), with minor modifications. Mycelia from control and (20mM) DTT-treated cultures were harvested and ground in liquid nitrogen. All further steps were performed on ice. The ground mycelia were resuspended (9g in 27 ml) in isolation buffer containing 10mM PIPES (pH 6.9), 0.5 M sucrose, 5mM CaCl₂, 5mM MgSO₄, 1mM PMSF, and 0.1 % 2-mercaptoethanol. The suspension was filtered through Miracloth. The filtered suspension was centrifuged for 10 minutes at 110g. The supernatant was centrifuged again to pellet the nuclei for 20 minutes at 4,000g. The pellet was resuspended in 1.5 ml isolation buffer and loaded to 6.5 ml of the same buffer, but containing 2.1 M sucrose, and centrifuged for 60 minutes at 161,000g. The pellet was resuspended in 0.25 ml of buffer containing 50mM Tris-HCl (pH 8.3), 40% glycerol, 5mM MgCl₂, 0.1 mM EDTA and immediately stored at -80°C. The isolated nuclei were checked under the fluorescence microscope after staining with ethidium bromide (Figure 5.1). The OD 260nm:280nm ratio in nuclear suspensions was 1.12-1.15 and the variation in OD 260nm was less than 5% in all nuclei suspensions.

5.2.2.2 Nuclear run-on transcription assay

Nuclear suspensions (100µl) were thawed on ice and mixed with 100µl reaction buffer containing 10mM Tris-HCl (pH 8.0), 300mM KCl, 5mM MgCl₂, 5mM DTT, 1mM each of ATP, GTP, CTP and 60µCi [α 32P] UTP. The reactions were allowed to run for 30 minutes at 30°C. The nuclear DNA was degraded by adding 5U RNase-free DNase (Ambion) for 5 minutes at room temperature. Next, SDS and NaCl were added to final concentrations of 1% (w/v) and 0.4M respectively. The mixture was extracted once with phenol/chloroform and once

with chloroform. The RNA was precipitated with one volume isopropanol and centrifuged for 10 minutes at 10,500g. The RNA pellet was dissolved in 50µl RNase-free water and cleaned through Spin columns-30 (Sigma) following the manufacturer's instructions. Labelled RNA was denatured by boiling for 2 minutes and immediately used for hybridisation.

5.2.2.3 Hybridisation of DNA dot blot

All DNA fragments were prepared by PCR using genomic DNA of *A. niger* transformant GUS64. A PCR product of each DNA fragment was gel extracted using QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's instructions. 1.5 µg DNA of each sample was alkali-denatured and spotted on Hybond N+ (Amersham). Spotted DNA samples were as follows: uidAI fragment corresponds to *uidA* open reading frame region +1 to +621 of the *E. coli uidA* gene (Schlaman *et al.*, 1994), where +1 refers to the A nucleotide in the translation start codon ATG, uidAII fragment corresponds to *uidA* open reading frame region +621 to +1232 bp, *bipA* and actin DNA fragments were the same as described in Chapter 2 section 2.9. The negative control (*PglaAN*) used was a DNA fragment of 609bp corresponding to -1402 to -2011 bp upstream of the *uidA* translation codon in the 2.032 kb *glaA* promoter fragment. Blots containing these DNA samples were pre-hybridised for 15 minutes at 65°C in a HYB-9 (Puregene) hybridisation solution. The denatured labelled RNA was added to the pre-hybridised blot and hybridisation was continued overnight.

5.2.2.4 Washing and exposing DNA dot blot

Following hybridisation, blots were washed twice in 2xSSC, 0.1% (w/v) SDS for 10 minutes. Blots were visualised and the band intensities quantified using a Fujifilm BAS2000 phosphorimaging system. Faint bands were observed in the negative control, but the background for each band was corrected by subtracting the signal generated from the negative control. Similar observation of low background generated from negative controls when carrying out nuclear run-on assays has been made in other studies (Gouka *et al.*, 1997b).

5.3 Results

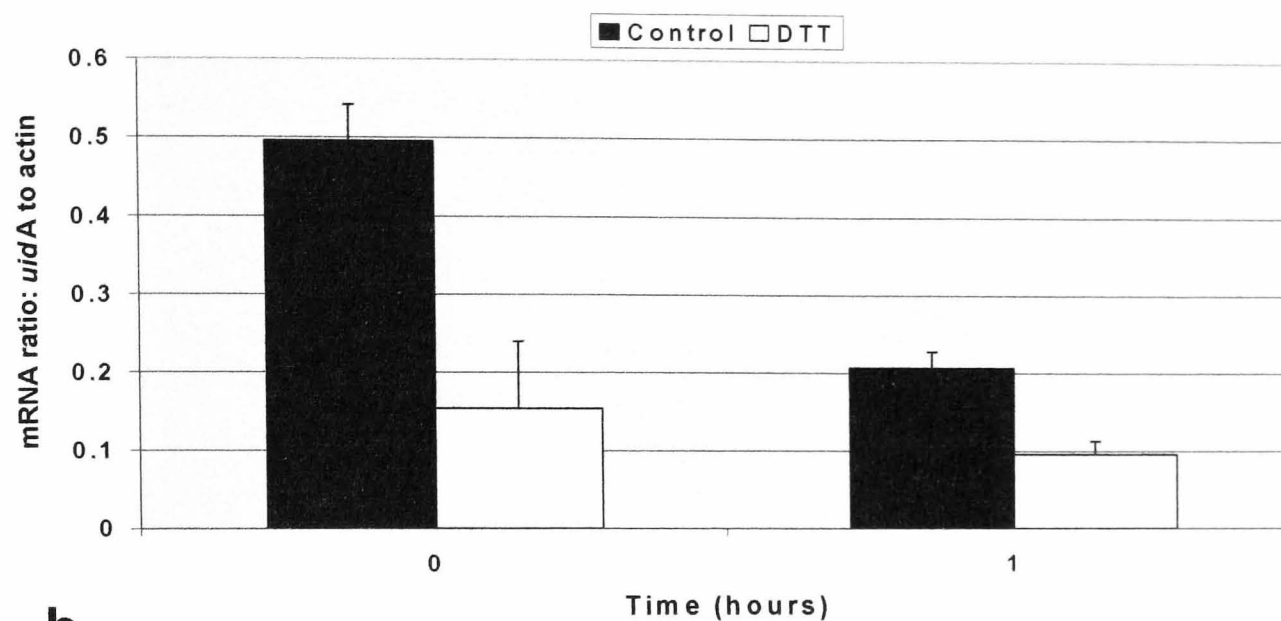
5.3.1 Transcription of *uidA* following medium-exchange

The results obtained in the previous Chapter (Figure 4.7) showed that in *A. niger* reporter strains GUS64*Sall*, GUS64*Bam*HI, and GUS64*Mlu*I the *uidA* transcript levels did not show an obvious increase, or decrease, during the time-course period used (6 hours) in these reporter strains. This would mean that the rate of *uidA* synthesis is similar to the rate of *uidA* degradation. For this reason the growth conditions needed to be optimised to improve the induction of the *uidA* gene to a level where an increase in the *uidA* transcript level is observed during the DTT time-course. To do so, media-exchange experiments were carried out to switch the 44 hours-old *A. niger* cultures grown in a medium with a *P_{glaA}* repressing carbon source (xylose) to a medium with an inducing carbon source. Figure 5.2 shows that the levels of *uidA* in the *A. niger* reporter strains decreased after 1 hour of medium-exchange, even in the control samples. These results suggest that one hour after medium-exchange is not long enough to start detecting a clear induction of the reporter gene. Furthermore, the pH was not adjusted in the medium into which the cultures were switched, to match the pH of the former media, which may result in a longer time needed for the cultures to adapt to the new medium. A time-course following the medium-exchange was performed next (as described in the Materials and Methods 5.2.1) to determine the time needed for *uidA* to start showing a detectable level of mRNA up-regulation. Figure 5.3 shows the *uidA* transcript levels over a time-course of 12 hours following the medium-exchange as detected by RPA. From this graph it can be seen that at least 6 hours following the medium-exchange is required to start detecting an increased level of *uidA*. Thus, 6 hours following the medium-exchange was considered as a 0 time-point before applying the target treatment (i.e. ER-stress triggered by DTT treatment). Furthermore, *bipA* transcript levels were detected (Figure 5.3 b) during the time-course in that experiment to monitor the ER-stress

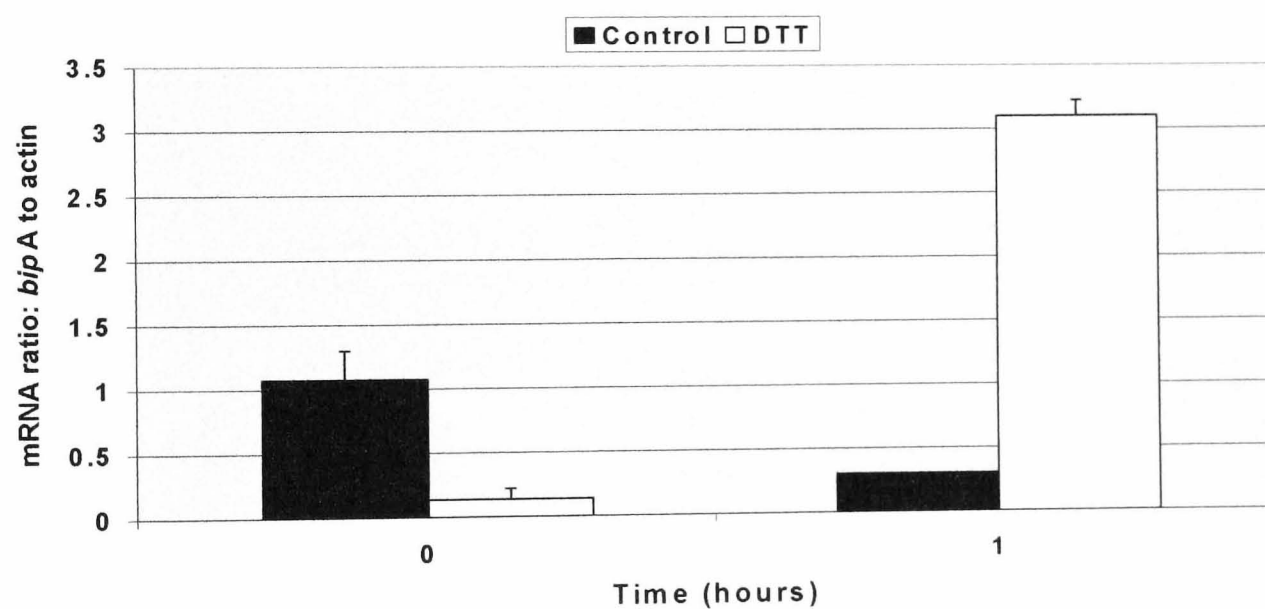
after changing the medium. No considerable up-regulation of *bipA* mRNA could be seen during the time-course, further confirming that no major ER-stress was taking place during the time-course as a result of exchanging the medium.

Figure 5.2 RPA of *uidA* reporter strains GUS64 and GUS64*SalI*. The DTT, or equivalent volume of sterile water (control) were added after medium-exchange (ACMX/N/P to ACMS/N/P), and mycelia were harvested immediately (0 time-point) and after 1 hour. The data represent averages from two replicates for each time-point. RPA probed for *uidA* (a and d), and *bipA* (b and e) in the same *uidA* reporter strains, GUS64 and GUS64*SalI*, respectively. c and f shows the RPA images that provide the quantified data presented.

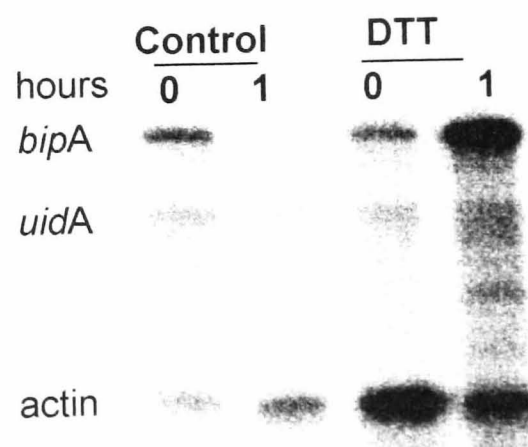
a. Transcripts levels of *uidA* under DTT treatment in GUS64 strain



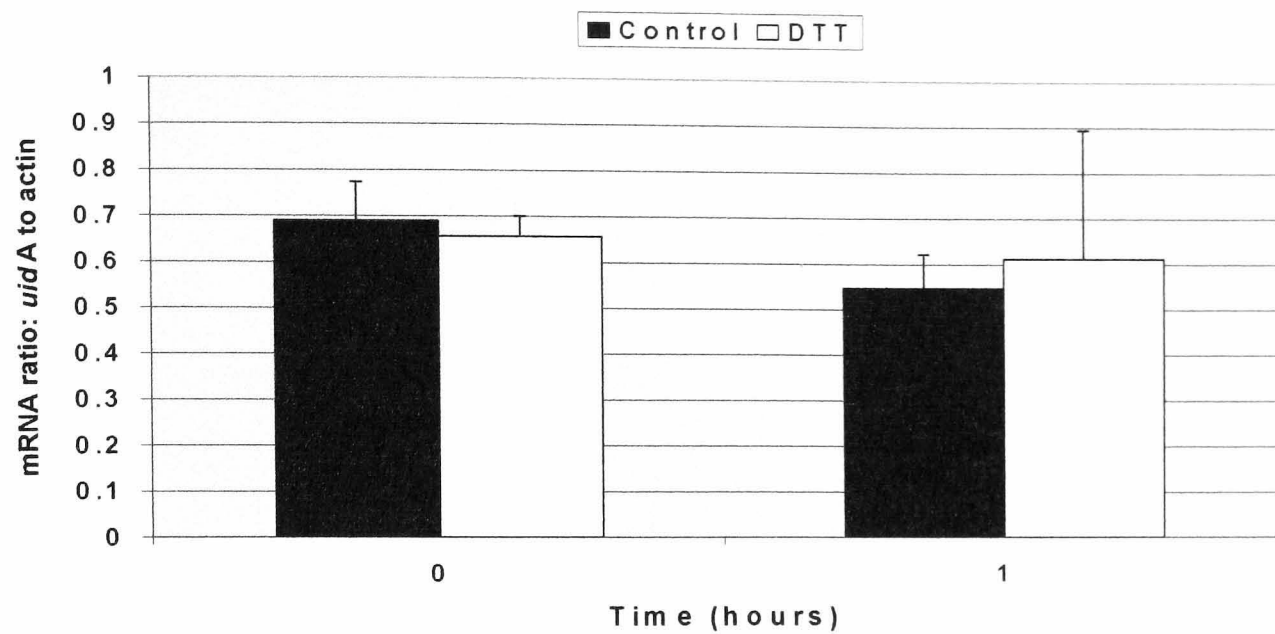
b. Transcripts levels of *bipA* under DTT treatment in GUS64 strain



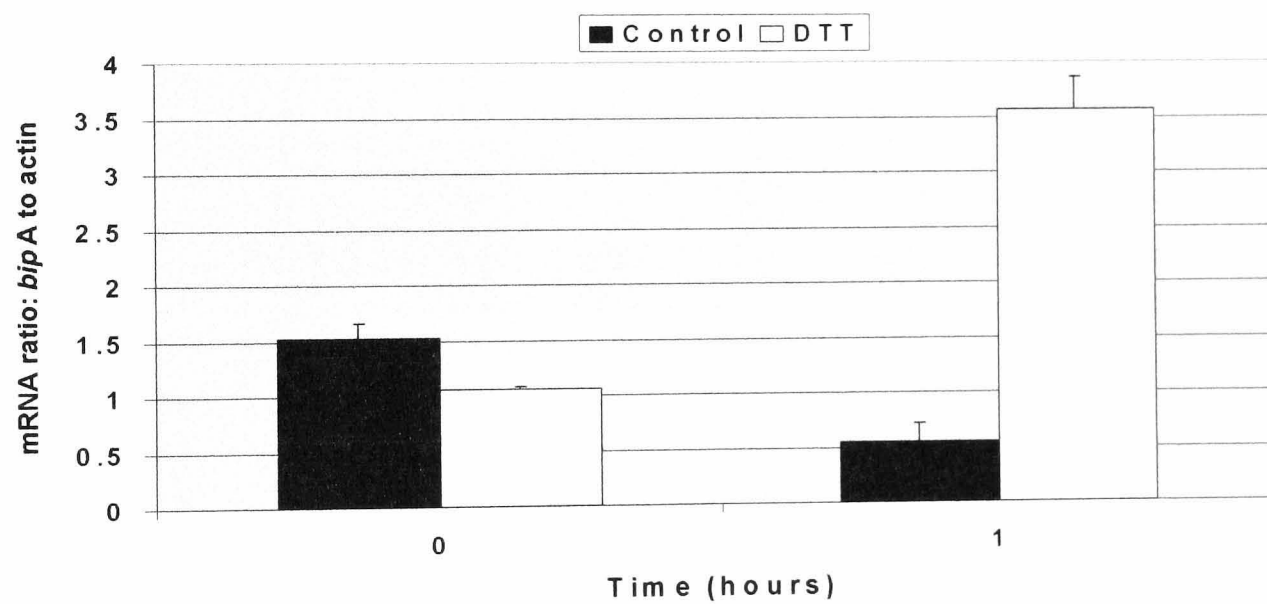
c.



d. Transcripts levels of *uidA* under DTT treatment in GUS64*Sal* strain



e. Transcripts levels of *bipA* under DTT treatment in GUS64*Sal* strain



f.

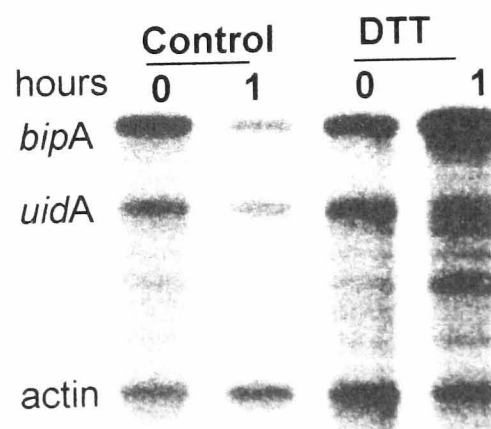
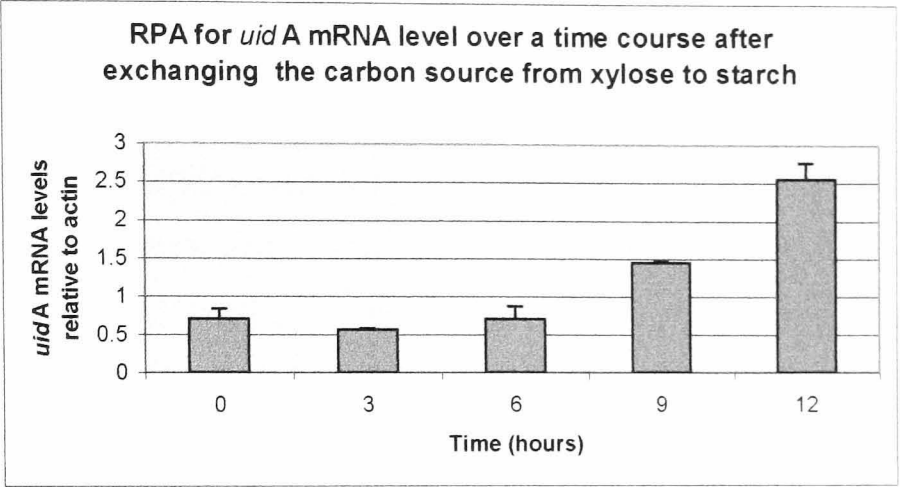
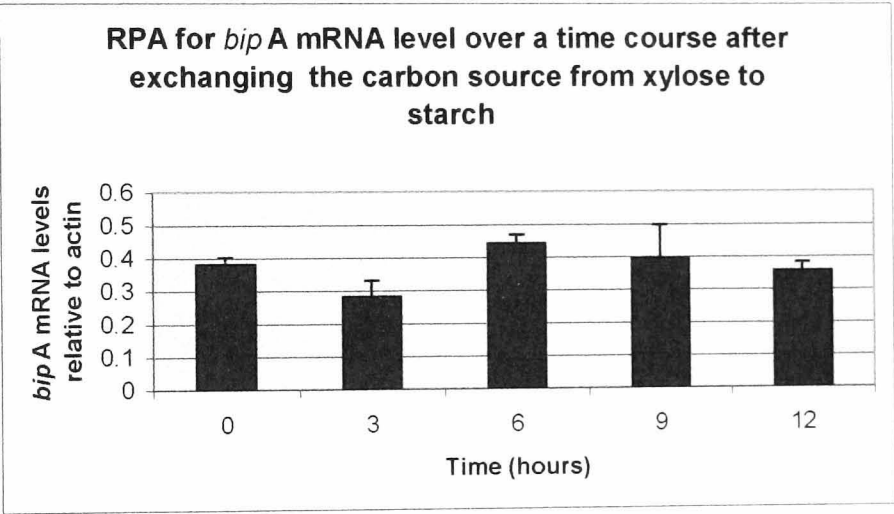


Figure 5.3 The relative transcript levels of *uidA* and *bipA* to actin transcript after medium-exchange in GUS64 strain. Panels a. and b. show the mRNA levels of *uidA* and *bipA* respectively. Panel c. shows the RPA image that provides the quantified data.

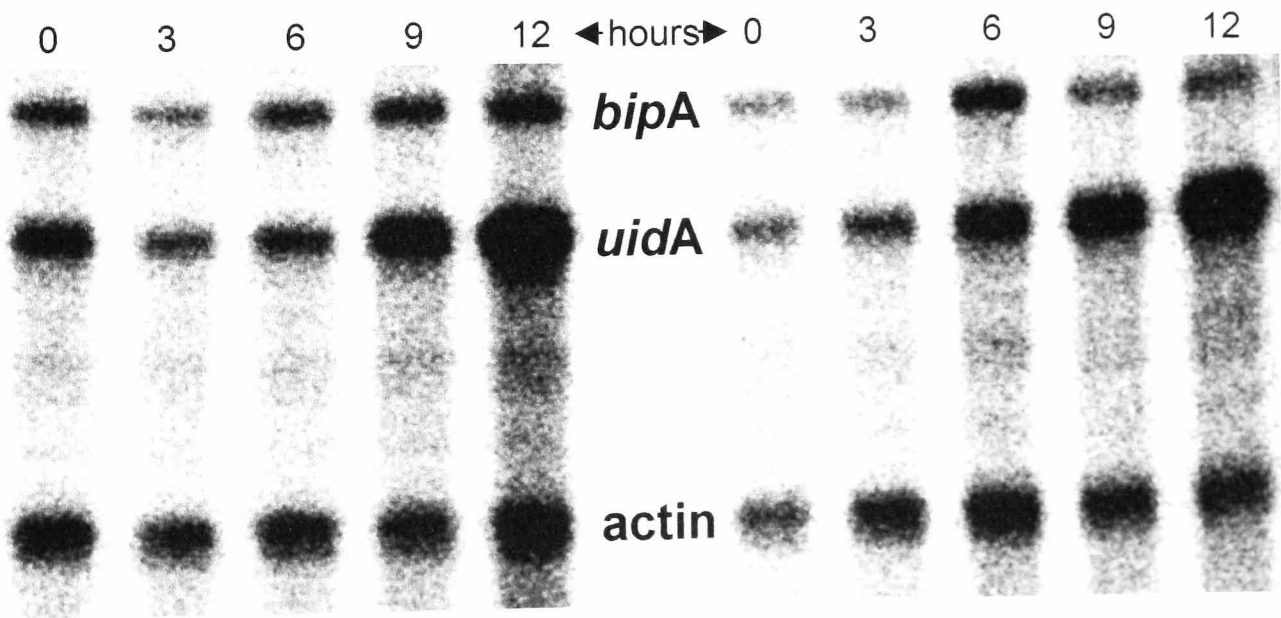
a.



b.



c.



5.3.2 Transcriptional regulation of the *uidA*-encoding gene in different *A. niger* reporter strains under ER-stress conditions

After finding the optimal time for detecting *uidA* induction, ER-stress experiments were carried out to initially check for the transcriptional down-regulation effect in the *A. niger* GUS64 and GUS64*SaI* strains. Since initial results (Chapter 4) showed that *A. niger* reporter strains GUS64*SaI*, GUS64*Bam*HI, and GUS64*Mlu*I had similar responses, in that none of them was able to mediate the down-regulation effect on *uidA* mRNA, the focus was limited to *A. niger* reporter strain GUS64*SaI*. Figure 5.4 (b) shows that the 2.032 kb P_{glaA} still exhibits the ability to down-regulate the *uidA* gene when the strain is exposed to DTT. The truncated P_{glaA} (1.195 kb) is not able to mediate the down-regulation effect (Figure 5.4 b), but the levels of the *uidA* mRNA are lower in this strain, showing that some promoter elements important for transcription are missing. In both instances, the levels of *bipA* mRNA were significantly increased in DTT-treated samples (Fig. 5.4 c), confirming that the UPR was active. The data presented in Figure 5.4 are from a single experiment, because the repeat experiment failed due to a degraded actin probe and time constraint meant that it could not be done again. However, the raw data (generated from probing with *bipA* and *uidA* probes only) showed the same qualitative trend (Figure 5.5) as the one in Figure 5.4. Results presented to this point show that the steady-state levels of the reporter *uidA* mRNAs were lowered when *A. niger* was stressed by DTT, only when the *uidA* reporter gene was expressed under the control of 2.032 kb P_{glaA} . This observation strongly suggests that the effect is transcriptional. However, to further confirm and validate this observation, the *de novo* mRNA of *uidA* was checked next, to eliminate any other possibility that may be responsible for this phenomenon.

Figure 5.4 RPA of *A. niger* GUS64 and GUS64SaII strains showing *uidA* transcripts (b) and *bipA* transcripts (c) relative to actin. Strains were treated with equal volumes of either water (black bars) or 20 mM DTT (white bars). The (a) panel shows the RPA images that provide the quantified data presented. Numbers in a panel represent the time in hours after addition of the DTT or the equivalent volume of water (control).

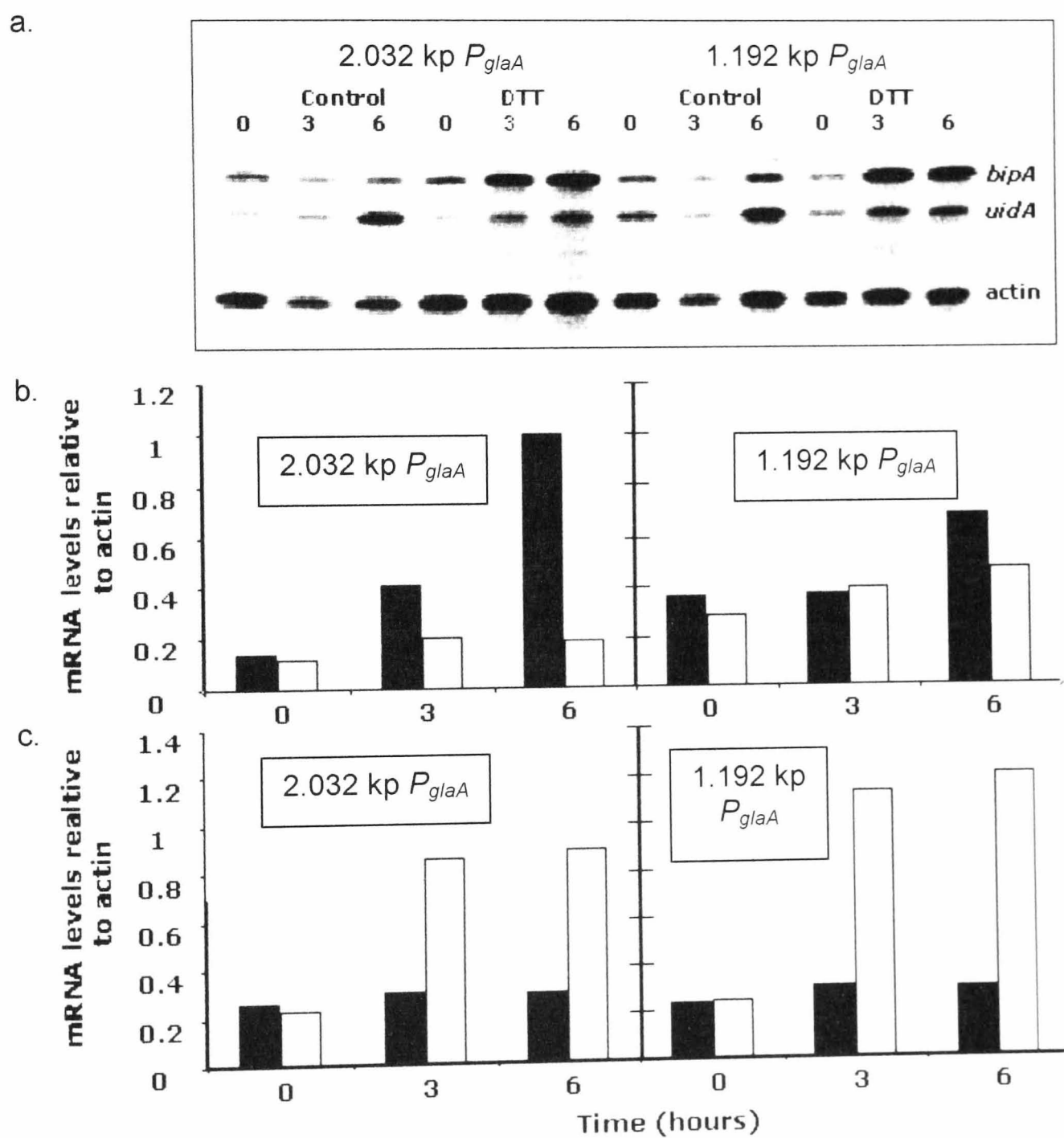
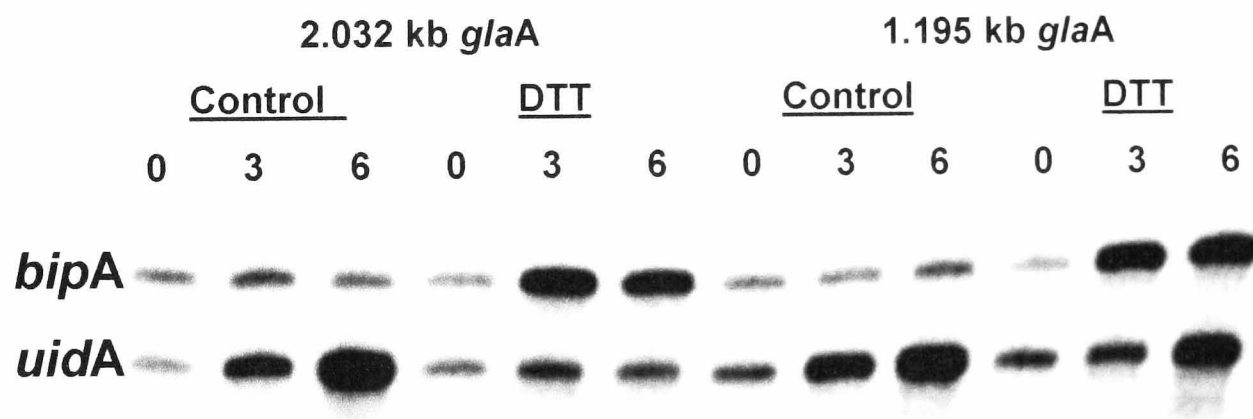


Figure 5.5 RPA for detecting the DTT effect on *uidA* in *A. niger* reporter GUS64 and GUS64*SalI* strains. Samples were probed with *bipA* and *uidA*. Numbers in the top of the image represent the time in hours after addition of the DTT or the equivalent volume of water (control).



5.3.3 The mechanism of transcriptional down-regulation under secretion stress is a feedback mechanism that functions at the transcriptional level

The nuclear run-on studies were applied to measure the impact of DTT specifically on transcription of *uidA* driven by P_{glaA} . The data in Figure 5.6 (I and II) show that 20 mM DTT generally suppresses transcription but that the impact is far greater on the *uidA* mRNA than on that of actin. The nuclear run-on results clearly showed that the transcriptional down-regulation effect is mediated by a region of P_{glaA} between 1.195 kb and 2.032 kb upstream of the translational start. UPR was confirmed in this system in response to the DTT treatment by probing for *bipA*.

The observation that DTT treatment generally suppresses transcription led to a check of the effect of DTT treatment on the viability of the cells following DTT treatment. The nuclear run-on experiments, carried out initially after 6 hours of DTT treatment, showed that actin could still be synthesised, indicating, indirectly, the viability of the cells (Figures 5.9). However, 6 hours of DTT treatment was found to significantly reduce the *de novo* mRNA levels of genes in the study - as detected by nuclear run-on experiments, possibly as a result of affecting the quality of the nuclei - and thus was avoided. Shorter exposure times were then used.

Repeating the nuclear run-on experiment after both 30 and 60 minutes of DTT treatment showed the same down-regulation effect. Furthermore, two different *uidA* fragments (*uidAI* and *uidAII*) were used in hybridisations to further confirm the observation and demonstrate elongation of the transcribed *uidA* mRNA. The results in Figure 5.6 (I and II) showed that the elongation of the *uidA* *de novo* mRNA was successful, as the *uidAII* probe showed similar transcription activities as *uidAI*.

Figure 5.6 Nuclear run-on for *uidA* and *bipA* 30 minutes and 60 minutes after treatment with either water (control - black bars) or 20 mM DTT (white bars). Data in each of the graphs are from one experiment. However, two *uidA* fragments (*uidAI* and *uidAII*) were used and the experiment was repeated at two different DTT treatment periods. Images at the bottom of the pages represent the nuclear run-on images that provide the quantified data.

uidAI: Corresponds to *uidA* ORF region from 1bp to 621bp, where 1 refers to the A nucleotide in the translation start codon ATG

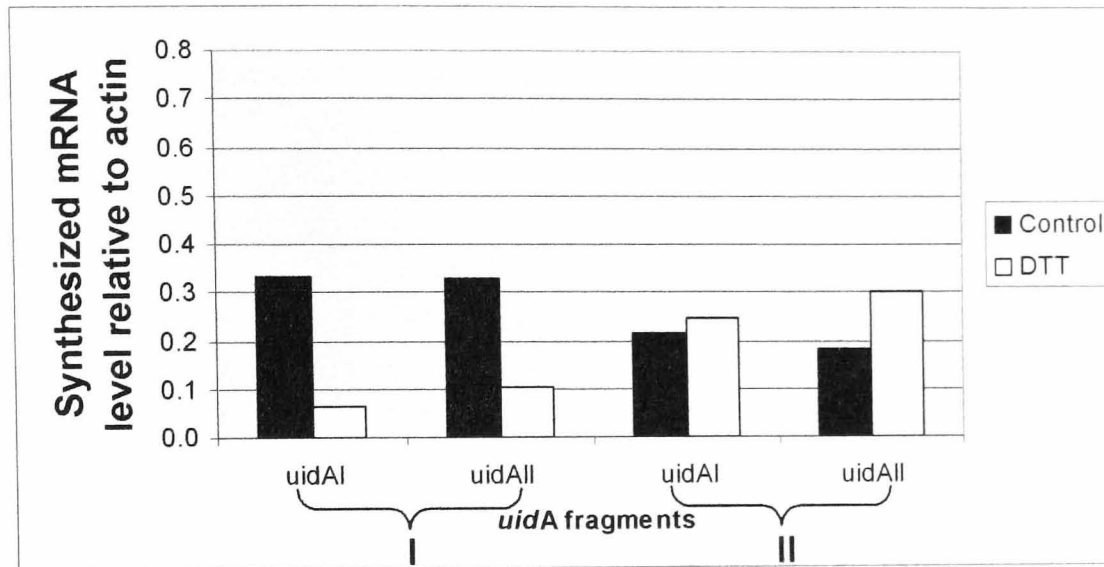
uidAII: Corresponds to *uidA* ORF region from 621bp to 1232bp from the A nucleotide in the ATG

I: *A. niger* GUS64 reporter strain

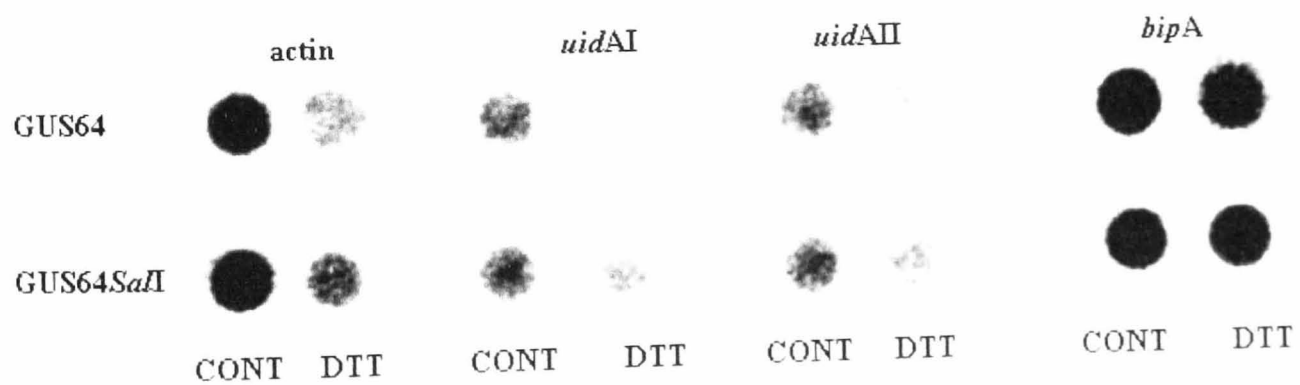
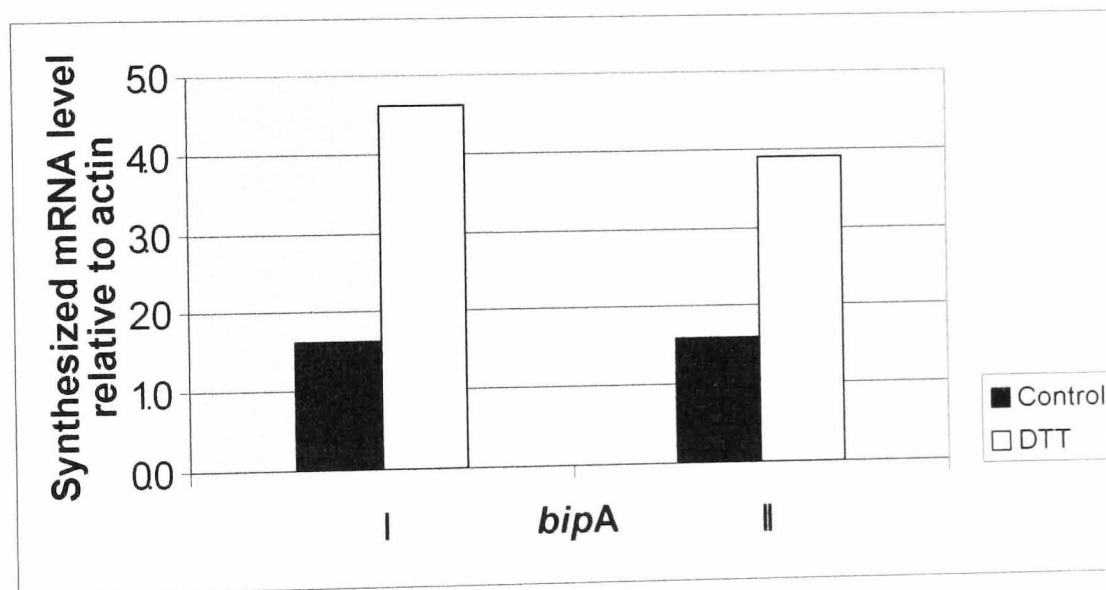
II: *A. niger* GUS64*SaII* reporter strain

CONT: water control

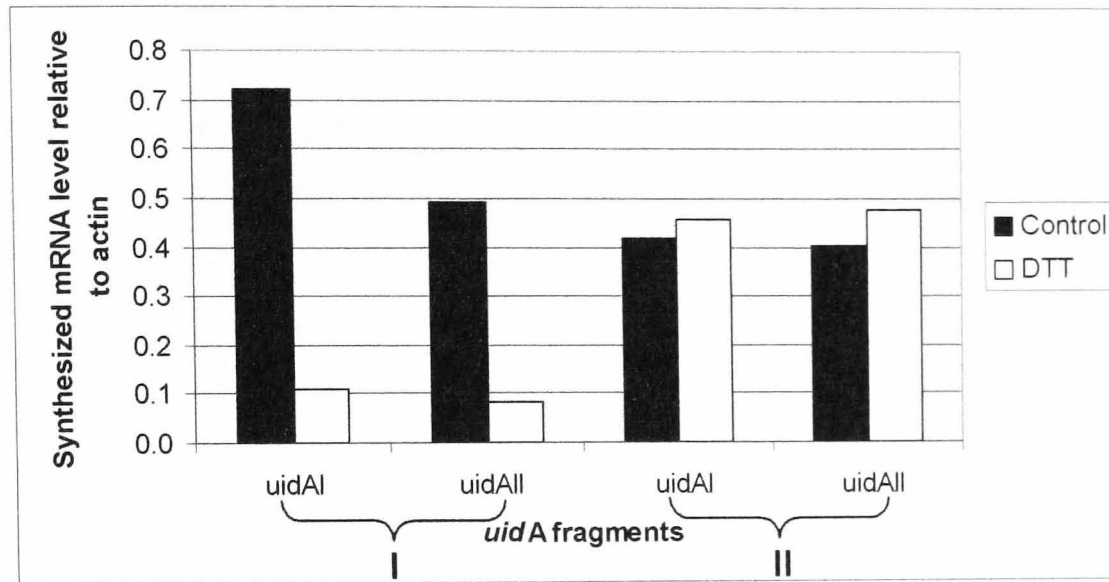
Nuclear run-on for *uidA* after 30 minutes of DTT treatment



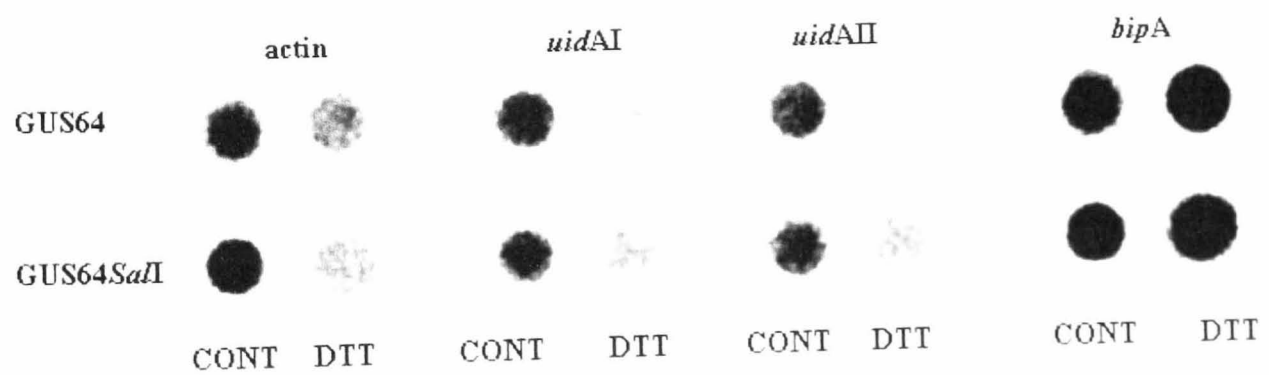
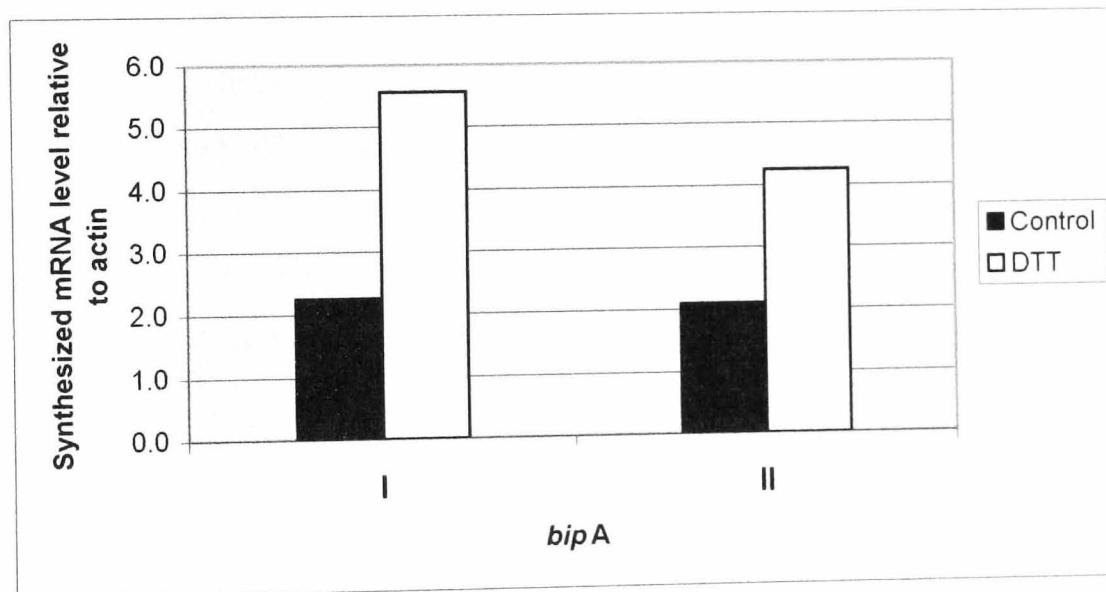
Nuclear run-on for *bipA* after 30 minutes of DTT treatment



Nuclear run-on for *uidA* after 60 minutes of DTT treatment



Nuclear run-on for *bipA* after 60 minutes of DTT treatment



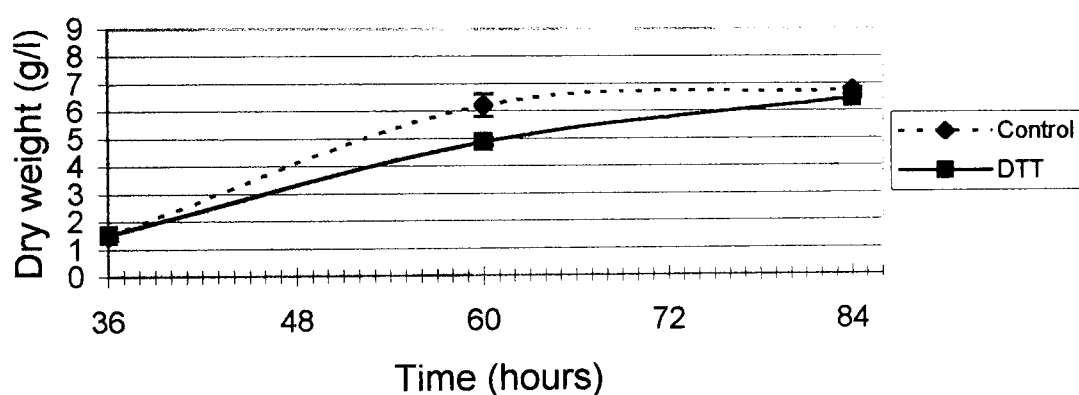
5.3.4 Effect of DTT treatment on the viability of *A. niger* cultures

One general effect of DTT on the transcription of genes in the study was to down-regulate transcription from the actin gene suggesting an inhibition of the growth of the fungus. The effect of adding DTT at the final concentration of 20 mM and 40 mM on the growth of *A. niger* GUS64 strain was examined. Also, growth of *A. niger* after DTT treatment for 1 and 6 hours was examined. Figure 5.7 (a and b) shows that, under these conditions, 20 mM DTT was not lethal, as the cells were able to grow after removal of DTT. The effect of 20mM DTT treatment for 6 hours was more severe on the mycelial growth than 1 hour of treatment. The reduction on the mycelial dry weight between the control and the DTT-treated sets of samples after 24 hours of post-DTT treatment for 6 hours was 31%, compared to 20% after 1 hour of DTT treatment. After 48 hours post-DTT treatment the effects on the cell dry weight were less pronounced. The reduction on the mycelial dry weight for the 6 hours DTT treated samples was 17%, compared to 4% for the 1 hour DTT treated samples. The important point in these observations is that neither the 1 nor 6 hours of 20mM treatment was lethal. However, the mycelia were not able to grow as long as DTT at a final concentration of either 20 mM or 40 mM was present in the media (Figure 5.8). Furthermore, there was a mycelial dry weight difference after 48 hours of DTT treatment at 20 mM or 40 mM (Figures 5.8 a and b respectively), indicating that 40 mM DTT was more toxic than 20 mM.

Figure 5.7 The effect of 20 mM post-DDT treatment on the growth of *A. niger*. *A. niger* GUS64 cultures were initially grown for 36 hours, before adding DTT to a final concentration of 20mM. After DTT treatment for 1 or 6 hours the medium was replaced with the same one, but without DTT. Time shown in the plot corresponds to the time after exchanging the medium. Standard errors generated from three replicates.

a.

DTT effect on the growth of *A. niger* GUS64 after 60 minutes treatment at a final concentration of 20mM DTT



b.

DTT effect on the growth of *A. niger* GUS64 after 360 minutes treatment at a final concentration of 20mM DTT

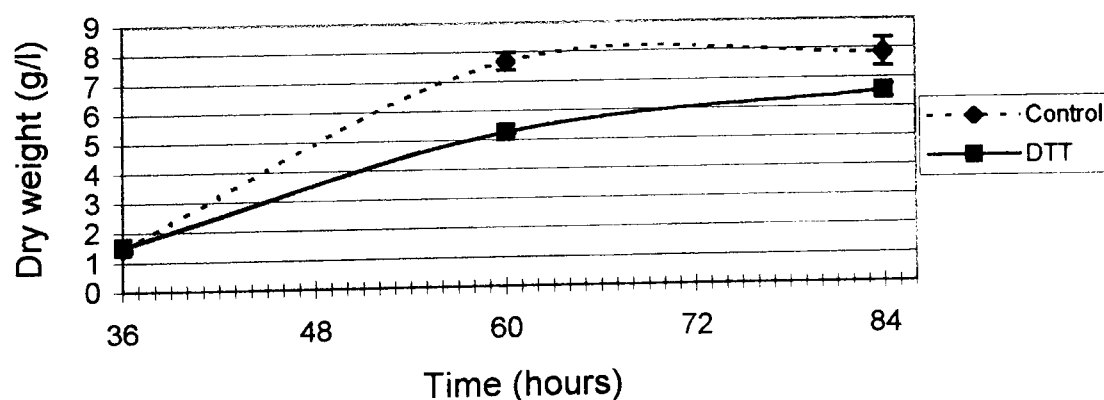
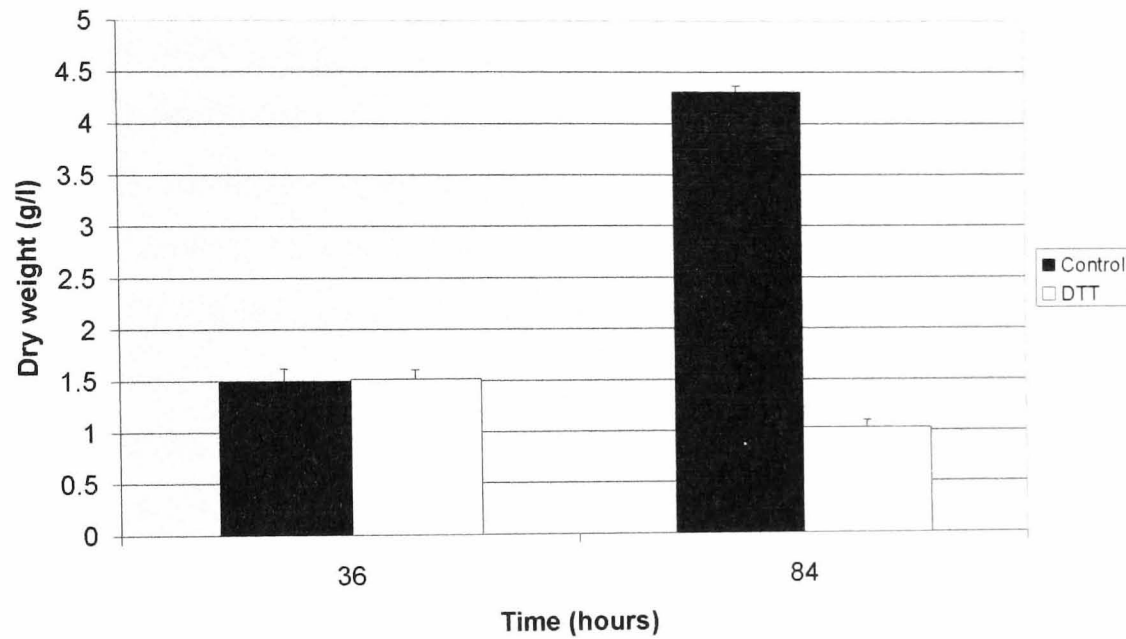


Figure 5.8 The effect of DTT on the growth of *A. niger* GUS64

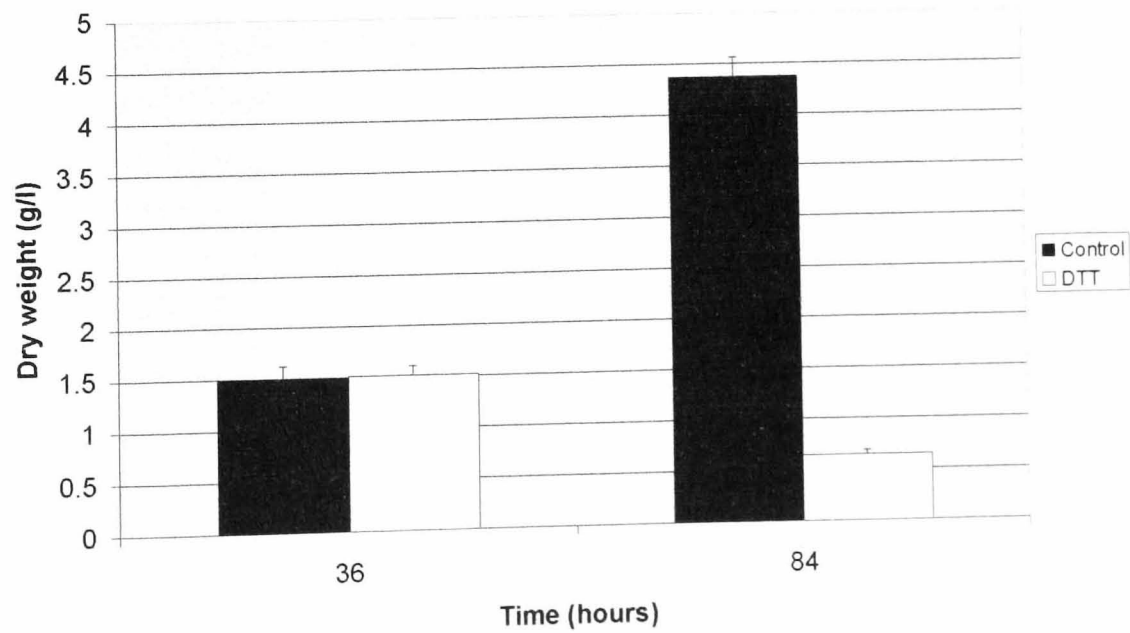
a

The effect of DTT on the growth of *A. niger* GUS64 at the final concentration of 20mM. DTT was added after an initial growth period of 36 hours



b

Growth of *A. niger* GUS64 at the final concentration of 40mM. DTT was added after an initial growth period of 36 hours

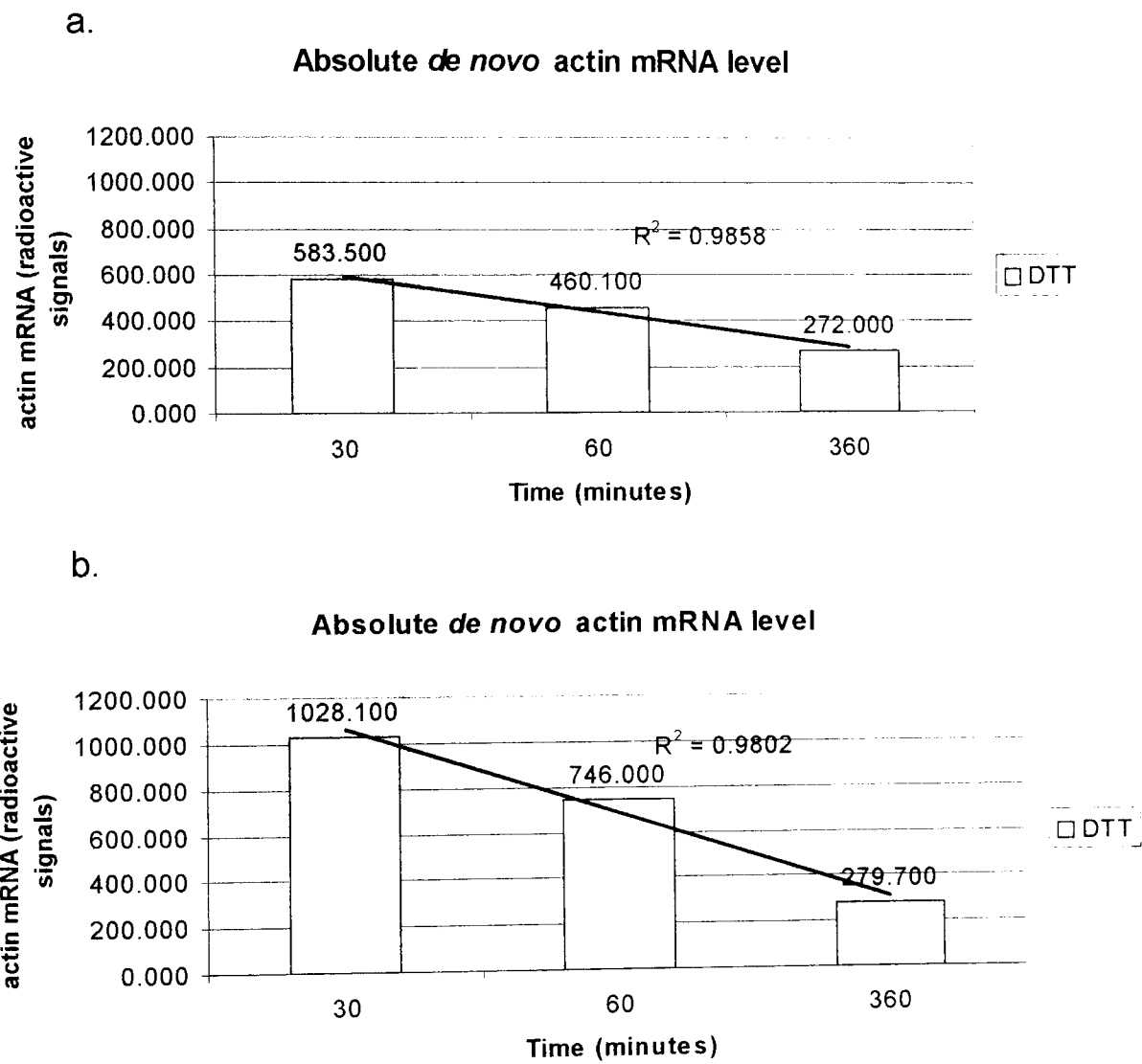


5.3.5 DTT leads to general transcriptional repression

The presence of DTT in the medium stops the growth of *A. niger* possibly as a result of arresting the cell cycle. It was interesting to compare the absolute *de novo* levels of actin mRNA, since transcription of actin is regulated by the cell cycle. Figure 5.9 shows that the longer the exposure time to DTT, the lower the *de novo* level of actin mRNA, as detected both in the GUS64 and GUS64*SaI* strains. The correlation between the reduction of *de novo* actin mRNA levels and the time of DTT treatment may suggest that no more new nuclei are being produced, as a result of stopping the growth (Figure 5.8), and thus lowering actin *de novo* levels. Although there is no direct evidence for cell cycle involvement, the given suggestion explains a possible scenario that may occur under DTT treatment conditions. A reduction in actin mRNA levels may not be detected by northern blots or RPA, which detect the total RNA, possibly due to the long half-life of actin mRNA as seen in mammalian cells. The β -actin mRNA half-life in mammalian cells as detected by Real-time RT-PCR was found to be 6.6-13.5 hours, depending on the cell line (Leclerc *et al.*, 2002). However, since actin is regulated as a function of the cell cycle it would still be present in proportion to the number of nuclei present in the sample in the study, and thus, as an internal standard, actin might be used for RNA loading normalization. However, the results generated in this study suggest that more research needs to be carried out to study how useful it is to use actin mRNA as an internal standard for RNA loading normalization in *A. niger*.

Figure 5.9 The absolute *de novo* level of actin mRNA detected by nuclear run-on experiments

- a. *A. niger* strain GUS64
- b. *A. niger* strain GUS64SaII



5.4 Discussion

The transcriptional down-regulation of the reporter gene under the control of P_{glaA} under ER stress has been demonstrated using both the RPA and nuclear run-on studies. The optimal time for detecting the up-regulation of *uidA* under the control of P_{glaA} following medium-exchange was determined. In Chapter 4 the *uidA* levels produced by *A. niger* reporter strains GUS64*SaI*, GUS64*Bam*HI, and GUS64*Mlu*I were similar throughout the time-course (Figure 4.7). However, after detecting the optimal time for *uidA* induction after medium-exchange, which was found to be 6 hours, an increase in the *uidA* mRNA during the time-course period could be detected (Figure 5.4). In a different study, where a gene fusion strategy was applied to study the P_{glaA} in *A. niger* using a reporter protein - green fluorescent protein (GFP) - it was found that, when shifting the samples from a non-inducing medium (containing xylose) to an inducing medium (containing maltose), the time delay between the medium shift and detection of the product expressed under the P_{glaA} was between 3 and 6 hours (Henriksen *et al.*, 1999). The time delay when carrying out a medium-exchange before detecting *glaA* induction seen in that study and the time delay found in my study confirms that about 6 hours is needed for the P_{glaA} to show strong induction when carrying out a medium-exchange. This might be critical when performing the nuclear run-on study on a reporter gene that is integrated as a single copy. In a previously-reported study carried out to examine the effects of a fusion of a truncated *glaA* gene on the mRNA levels for the gene encoding the guar α -galactosidase (*aglA*) in *A. niger* under the control of the 1,4- β -endoxylanase A promoter, it was found that the mRNA levels of an *aglA* single-copy strain were too low to carry out reliable run-on assays. Instead, a multicopy strain, which had a higher *aglA* mRNA level, was used (Gouka *et al.*, 1997b). Improving the *A. niger* growth conditions for detecting the transcripts of the target gene by nuclear run-on experiments may have helped in detection.

As the medium-exchange experiments involve switching the cells to a new environment, several factors could potentially subject the cells to stress, such as the medium pH and temperature. In such a case, false results could be generated

regarding gene expression. For this reason, *bipA* RNA was monitored throughout the time-course following medium-exchange experiments. It is possible that changes in *bipA* mRNA levels may not indicate every environmental stress the cell may encounter during or after the medium-exchange, but at least under ER-stress conditions it is known that the *bipA* gene in *A. niger* is up-regulated (van Gemeren *et al.*, 1997).

In this Chapter the transcriptional down-regulation effect is shown by nuclear run-on studies to be at the level of transcription. Furthermore, this mechanism has been confirmed to be mediated through the promoter of *glaA* in a region more than 1kb upstream of the translational start. These observations lead to the question, mainly: how can the transcription of the *glaA* gene be controlled from a large distance in the P_{glaA} ? Also, is the transcriptional down-regulation mechanism likely to be mediated separately from the UPR or not? These questions are discussed in Chapter 6.

The apparent transcriptional down-regulation of genes encoding secreted proteins has also been observed in *T. reesei* (Pakula *et al.*, 2003), where it was termed RESS (repression under secretion stress), and in *A. thaliana* (Martinez and Chrispeels, 2003). Martinez and Chrispeels (2003) demonstrated in a global analysis of gene expression under separate DTT and tunicamycin stress that most of the genes that were down-regulated contained signal sequences targeting the proteins to the cell wall or to extracellular locations. Furthermore, Pakula *et al.* (2003) suggest that the down-regulation of cellulase (a secreted protein) transcripts during secretion stress seems to be distinct from the *cre1*-mediated glucose repression system (Ilmén *et al.*, 1996; Ilmén *et al.*, 1997). Their suggestion was based on the observation that *T. reesei* strains, either with a wild type or defective *cre1* glucose repression system, display down-regulation of the cellulase transcripts in response to secretion stress to the same extent. Based on their results, it is not known whether the RESS directly involves components of the UPR regulatory pathway or not. A number of indirect observations in their study may suggest that RESS and UPR mechanisms may be mediated differently. These observations include the severe down-regulation of cellulase mRNA in

cultures treated with a calcium ionophore A23187, which affects the permeability of the cellular membrane to Ca^{2+} ions and depletes the intracellular stores of Ca^{2+} (Brostrom *et al.*, 1989; Wileman *et al.*, 1991), while this drug only had a slight effect on UPR. Similarly, tunicamycin treatment resulted in cellulase down-regulation “although tunicamycin is a poor inducer of UPR in the fungal strain” (Pakula *et al.*, 2003). Moreover, the *cbh1* promoter does not contain consensus UPR elements, and thus the effect is unlikely to be mediated by HAC1 (Pakula *et al.*, 2003). In my study, the transcriptional down-regulation of the *glaA*-encoding gene in the absence of UPR, using a strain with a functionally incomplete protein folding system (AS1.1) (Figure 3.9), would further support the independence in mediating these two mechanisms, transcriptional down-regulation and UPR.

Generally, in yeast and mammalian cells, the promoters of the UPR-induced genes are characterised by specific response elements: yeast UPRE, CAGCGTG; mammalian UPRE, TGACGTGG/A; mammalian ERSE-I, CCAAT-N₉-CCACG, and mammalian ERSE-II, ATTGG-N-CCACG. However, for down-regulated genes under UPR activation, no specific response elements have been characterised yet. In *A. thaliana* Martinez and Chrispeels (2003) performed sequence analysis on the promoters of the UPR down-regulated genes (129 genes) identified in their study using MEME (<http://meme.sdsc.edu>) or the Motif Sampler program (<http://www.esat.kuleuven.ac.be/~dna/biol/Software.html>). In their analysis study, 1 kb of up-stream sequences from known or predicted translation start codons of these UPR down-regulated genes were searched, but no statistically significant motif in the promoters of these genes was found. This finding does not necessarily mean that there is no promoter element(s) that mediates the transcriptional down-regulation of these genes. One possibility could be that the element(s) might be located further up-stream of the 1 kb region.

In conclusion, this study presents a new ER-associated stress response in the filamentous fungus *A. niger* that has been termed transcriptional down-regulation (Al-Sheikh *et al.*, 2004). This mechanism selectively down-regulates the gene encoding glucoamylase, but not the non-secreted protein, actin. Also, it is the first study to show clearly that the effect is transcriptional, rather than being

based on steady-state transcript levels. Finally, it has been shown in this study that by subjecting *A. niger* to ER-stress conditions triggered by DTT treatment, the UPR (Chapter 3), ERAD (Chapter 3), and transcriptional down-regulation (Chapter 5) mechanisms were all activated. Thus, the UPR, ERAD and transcriptional down-regulation mechanisms may all work together to help the cell to cope with the stress, by improving protein folding and transport, degrading unwanted proteins, and allowing fewer secretory proteins to enter the ER, respectively.

6 General discussion

Filamentous fungi are widespread in nature and include many species that are saprophytic. Many filamentous fungi species secrete a wide variety of enzymes capable of degrading a large diversity of biopolymers (Oxenboll, 1994). The ability of many filamentous fungi such as *A. niger* and *T. reesei* to secrete a high level of different enzymes has stimulated the industrial application of these species (Archer and Peberdy, 1997; van Gorcom *et al.*, 1994). However, inefficient transit of proteins, whether homologous or heterologous, destined for secretion through the ER, could be a significant bottleneck in production of commercially viable levels of protein (Conesa *et al.*, 2001a).

The inhibition of protein folding within the ER leads to cellular responses, collectively called ER stress, and the UPR is the cellular response that has been studied most extensively so far (Mori, 2000a; Patil and Walter, 2001). Previous studies have shown that the UPR is active in *A. niger* (Ngiam *et al.*, 1997; Ngiam *et al.*, 2000; van Gemeren *et al.*, 1997) and is induced by DTT and by the secretion of heterologous proteins (t-PA) (Mulder *et al.*, 2004). Similar effects have also been shown in *T. reesei*, producing the heterologous protein IgG antibody Fab fragment (Saloheimo *et al.*, 1999).

In this study several findings have been reported. The main findings include:

(a) Subjecting *A. niger* to ER-stress conditions triggered by DTT treatment, the UPR (Chapter 3), ERAD (Chapter 3), and transcriptional down-regulation (Chapter 5) mechanisms were all activated.

(b) The new ER-associated stress response that has been reported in this study in *A. niger*, which is termed transcriptional down-regulation, selectively down-regulates the gene encoding glucoamylase, but not the non-secreted protein, actin.

(c) This is the first study to show clearly, by nuclear run-on studies, that the effect is transcriptional, rather than being based on steady-state transcript levels.

(d) The transcriptional down-regulation was found to be mediated through the promoter of *glaA* in a region more than 1.192 kb upstream of the translational start.

(e) Preliminary studies using antisense *pdiA* strains of *A. niger* suggest that the transcriptional down-regulation of genes encoding secreted proteins and UPR are likely to be mediated by different signalling pathways.

(f) Tunicamycin is a poor ER-stress agent compared to DTT, and other factors such as the pH, the age of the cultures, and the solvent agent may need to be considered when using tunicamycin as a stress agent to stress *A. niger* cultures.

(g) The UPR is not obviously activated in *A. niger* B1 strain that overexpresses HEWL protein in ACMS/N/P medium.

It has been shown that, under ER-stress conditions triggered by DTT treatment in *A. niger*, ERAD is also activated (Chapter 3). Although the sensor involved in ERAD is not known, research suggests that the UPR and ERAD are very closely integrated in yeast (Travers *et al.*, 2000). The activation of the ERAD response mechanism in *A. niger* was demonstrated by the up-regulation of a gene (*rpnG*) encoding the 26S proteasomal regulatory subunit, which is involved in degrading incorrectly-folded proteins, including those from the endoplasmic reticulum (ER). The *A. niger* *rpnG* is a homologue of the *S. cerevisiae* *rpn7*. At the present time, the only 26S proteasomal subunit gene identified from *A. niger* is one which encodes a homologue of the yeast regulatory particle Rpt3p subunit (TbpA), which is a member of the AAA-ATPase family (Jarai, G., unpublished data; Swiss-Prot P78578, MacKenzie, personal communication). Recent studies on a yeast *rpn7* mutant have suggested that the Rpn7p subunit plays a crucial role in forming the correct lid structure of the regulatory particle and, hence, in maintaining the integrity of the 26S proteasomal complex (Isono *et al.*, 2004). In Chapter 3 it was shown that treatment of *A. niger* with DTT stimulated transcription of *rpnG* by approximately threefold. A similar three fold level of induction was

observed with the *T. reesei prs12* gene that encodes another 19S regulatory particle non-ATPase subunit, the homologue of the yeast Rpn8p, in response to the reducing agent, β -mercaptoethanol (Goller *et al.*, 1998). However, these results contrast with the situation in *S. cerevisiae*, where at least some 19S proteasomal subunit genes, e.g. *rpn7*, *rpn8* and *rpn9*, were not classed as UPR target genes (Travers *et al.*, 2000), although this may reflect differences in regulation between yeast and filamentous fungi. Furthermore, UPR is essential for ERAD. Gene expression analysis studies reveal an intimate coordination between these responses: efficient ERAD requires an intact UPR, and UPR induction increases ERAD capacity. On the other hand, loss of ERAD leads to constitutive UPR induction (Travers *et al.*, 2000).

The transcriptional down-regulation effect is shown by nuclear run-on studies to be at the level of transcription, rather than mRNA stability, and is found to be mediated through the P_{glaA} in a region more than 1.192 kb upstream of the translational start up to 2.032 kb. Use of a 1.192 kb P_{glaA} leads to a significant reduction in the level of gene expression using the *uidA* reporter strains compared to the un-truncated 2.032 P_{glaA} (Table 4.2). These observations raise several questions. First, has it been observed before that a fungal gene is regulated by a promoter element(s) located over a large distance from the translation site of that gene? Second, physiologically what could possibly be the significance(s) of the transcriptional down-regulation mechanism? Last but not least, how could the regulatory sequence communicate with the promoter over a large distance? Upstream regulatory sequences (URSs) located over a large distance from the transcriptional start site have been reported previously in the filamentous fungus *N. crassa*. Deletion analysis of the glutamate dehydrogenase encoding-gene (*am*) promoter sequence has identified two upstream regulatory sequences that control the level of gene expression. The closer of these two elements (URS α) is located approximately 1.4 kb upstream of the transcriptional start site. The second element (URS β) is located between 2.1 and 3.2 kb upstream of the translation start site. Deletion of either of these two elements reduces *am* expression to about 50% of the wild type level (Frederick and Kinsey, 1990). It was concluded in

the study that these elements behave more like weak enhancer elements (that enhance transcription but are not essential for it) than the upstream activation sites (UASs) elements of yeast (see below), since the complete removal of these two elements did not completely abolish the transcription of the gene. Later on, it was shown by a different study that a heteromeric nuclear protein, Am Alpha Binding protein (AAB-1), binds specifically to a CCAAT box within the URS α element, and that the CCAAT motif is essential for binding of AAB (Chen *et al.*, 1998; Chen and Kinsey, 1995). Disruption of the gene encoding AAB-1 results in a 50% decrease in *am* expression, as well as other unrelated effects on growth and differentiation. Furthermore, sequence alignment showed that AAB-1 is a homologue of the yeast HAP5 subunit of the heteromeric CCAAT binding protein complex (McNabb *et al.*, 1995). In *A. niger* P_{glaA} , Electrophoretic Mobility Shift Assay (EMSA) and DNase I footprinting analysis revealed that two CCAAT elements named DC and PC could competitively bind to a protein extracted from starch-induced mycelia, named AngCP (Qiu *et al.*, 2002). In a recent study on *A. niger* it was suggested that a DNA-protein loop was formed between AngCP, DC, and PC so that the DNA-protein loop may contribute to the transcriptional regulation of *A. niger* P_{glaA} (Zhu *et al.*, 2004). However, whether the CCAAT has any effect on the regulation of P_{glaA} under ER-stress conditions is not known. Nor is it known whether the CCAAT sequence in *A. niger* P_{glaA} used in this study, present at 1998-1994 bp upstream of the translation start site (Figure 4.1), plays any role in enhancing the transcription of *glaA*, although the deletion of the sequence above 1 kb (which contains the CCAAT sequence) results in a ca. 42% decrease in *glaA* mRNA level (Table 4.2).

The next question was what could be the physiological significance(s) of the transcriptional down-regulation mechanism in *A. niger*? The saprophytic filamentous fungi secrete enzymes to degrade organic polymers and it is possible that the transcriptional down-regulation mechanism has evolved in these organisms as a specific defence against unfolded protein stress due to the high flux of proteins destined for secretion through the ER. Confirmation that the transcriptional down-regulation mechanism is operating at the transcriptional level

would validate the suggestion that the transcriptional down-regulation of genes encoding secretory proteins results in lower protein input in the ER.

The apparent decline in the transcripts of genes encoding secreted proteins has also been observed in *T. reesei* (Pakula *et al.*, 2003) and in *A. thaliana* (Martinez and Chrispeels, 2003). Pakula *et al.* (2003) have reported their study in *T. reesei* subjected to different ER-stress agents. In their study the effects of DTT, Brefeldin (BFA), which in many cell types disrupts the structure of the Golgi compartment (Klausner *et al.*, 1992) and Ca^{++} -ionophore A23187 on total protein synthesis and secretion were reported. Also, proteomic studies using 2-dimensional (D) gel electrophoresis and northern analysis of several genes encoding secreted proteins were performed. The secretion rate of total labeled proteins into the culture medium, measured as the increase in the amount of total labeled protein in the culture medium per time unit, was efficiently inhibited in the presence of DTT or BFA and was only 5% of that in the control cells. These results may suggest the global effect of down-regulation on genes encoding secreted proteins under ER-stress conditions. This observation was further confirmed by northern analysis of several mRNAs of genes encoding extra-cellular proteins in DTT treated cells. These genes included *cbh2* (cellobiohydrolase), *egl2* (endoglucanase 2), *xyn1* (xylanase) and *hfb2* (hydrophobin). So these results indicate that many of the genes coding for extra-cellular proteins are subjected to a feedback-type control under conditions when such proteins would be unlikely to fold correctly. However, this was not seen in the case of genes coding for intra-cellular proteins, such as glyceraldehyde-3-phosphate dehydrogenase (*gpd1*) or aminobutyrate transaminase (*abt1*), where their mRNAs remained unchanged. Since this feedback-type control is selective on genes coding for extra-cellular proteins, it is very likely to be mediated by the transcriptional down-regulation mechanism characterised in this study. The finding reported in my study, that the transcriptional down-regulation mechanism is mediated by P_{glaA} , would suggest the possibility of promoter element(s) in these genes that mediate their down-regulation. Finally, the kinetics of synthesis and secretion of one secreted protein, CBHI, was investigated using 2D gels (Pakula *et*

al., 2003). In the DTT and BFA treated cultures, the average synthesis time of full-length CBHI was not significantly affected under each drug treatment (4.3 and 4.6 minutes, respectively) compared to the untreated cells (5.7 minutes). However, the minimum secretion time of CBHI measured in the BFA treated cultures was significantly longer (69 minutes) compared to the untreated cultures (11 minutes). In the DTT treated cultures this parameter could not be determined due to significantly low amounts of extra-cellular protein produced in these cultures. The final DTT concentration used in that study was 10 mM, compared to 20 mM used in my study. This may suggest that the use of 10 mM final concentration of DTT is enough to subject the cultures of *A. niger* to ER-stress. In mammalian and yeast cells 2 mM was enough to activate the UPR (Benedetti *et al.*, 2000; Travers *et al.*, 2000). Although the use of 20 mM DTT final concentration to treat *A. niger* cultures for 6 hours was not lethal (Chapter 5), it might be better to apply the minimum amount of DTT which has to be determined experimentally, to avoid the toxicity of DTT as much as possible. A further observation that may indicate the strong effect of DTT as a stress agent was that the rate of CBHI synthesis (the amount of radioactive labelled CBHI synthesised per time unit) as studied by 2D gels was 14% of that measured for the untreated cultures, whereas in the BFA it was 52% of that measured for the untreated cultures (Pakula *et al.*, 2003). The effects of the Ca^{++} -ionophore A23187 on total protein synthesis and secretion was less pronounced than the other stress agents (DTT and BFA) (Pakula *et al.*, 2003).

The apparent decline in the transcripts of genes encoding secreted proteins has also been observed in *A. thaliana* exposed to either DTT or tunicamycin (Martinez and Chrispeels, 2003). Gene expression analysis with Affymetrix GeneChips suggests that under ER-stress conditions that activate the UPR in *A. thaliana* at least 0.7 and 1.8% of the genome is up-regulated and down-regulated, respectively. Most of the genes that were down-regulated contained signal sequences targeting the proteins to the cell wall or to extracellular locations. Thus, the selectivity in down-regulating these genes may suggest the presence of a transcriptional down-regulation mechanism in *A. thaliana*. However, sequence analysis of 1 kb promoter regions from the known or predicated translation start

codons of genes that were down-regulated during the UPR showed no statistically significant motif in these promoters. These results may not conclusively prove that no promoter element was involved in mediating the down-regulation of these genes, for several reasons. Some of these reasons may include the possibility that the promoter region(s) that could be involved in the transcriptional down-regulation of these genes is located further upstream of 1 kb on the target promoters from the translation start codons of these genes. Also, it could be that the computer software packages used (MEME (<http://meme.sdsc.edu>) and the Motif Sampler program (<http://www.esat.kuleuven.ac.be/~dna/biol/Software.html>)) are not precise enough to specifically detect the promoter element(s) involved in the down-regulation phenomenon. To carry out such analysis it might be more efficient to perform it in smaller DNA sequences. This would help to first narrow down the promoter region(s) that might be involved in this down-regulation phenomenon.

The UPR has been well characterised in both yeast and mammalian systems. DNA microarray data of *S. cerevisiae* cultures treated with DTT or tunicamycin and strains expressing a heterologous protein (major histocompatibility complex class I heavy chain) showed examples of genes encoding secreted proteins that were down-regulated as well as those that are not affected under the conditions studied (Casagrande *et al.*, 2000; Travers *et al.*, 2000). In *S. cerevisiae* strains with secretory mutations the decreased transcription of genes encoding ribosomal DNA, ribosomal protein (RP) and tRNAs has been reported (Li *et al.*, 2000). In mammalian systems the UPR can be controlled at the translational level as well, a feedback response mediated mainly by the protein PERK (Harding *et al.*, 1999). PERK pathway activation leads to two secondary effects. First, the transcription factor ATF4 is up-regulated and induces the amino acid biosynthesis as it binds to an amino acid response element (AARE) (Harding *et al.*, 2000). ATF4 is a member of the ATF/CREB proteins that include CREB (cAMP responsive element binding protein) that binds to DNA via their basic region and dimerize via their leucine zipper domain to form a large variety of homodimers and/or heterodimers that allow the cell to coordinate

signals from different pathways (Hai *et al.*, 1989). Second, secondary effects of PERK pathway activation include selective stimulation of the m(7)G-cap-independent translation mechanism of mRNAs that contain internal ribosome entry sites within their 5' untranslated regions, such as cationic amino acid transporter cat-1 (Fernandez *et al.*, 2002). Interestingly, the amino terminus of the luminal domain of PERK shows limited homology to the amino terminus of the IRE1 luminal domain. The human IRE1 and PERK luminal domains can substitute for the *S. cerevisiae* IRE1 luminal domain to signal the UPR in *S. cerevisiae* (Liu *et al.*, 2000). This may suggest that both kinases are activated in parallel through a common mechanism. It has been demonstrated that PERK is sufficient to mediate loss of cyclin D1, a product of PRAD1 (parathyroid adenomatosis 1) also known as BCL1 which is believed to be required for progression into S-phase, and to promote cell-cycle arrest in G1 phase (Rosenberg *et al.*, 1991), and prevent cells from progressing through the cell cycle (Brewer and Diehl, 2000). This delay may allow the cell to pause in the cell cycle to determine whether adaptation to stressful conditions is possible by UPR or to continue to apoptosis (Niwa and Walter, 2000). Neither the existence of a functional PERK homologue nor attenuation of translation in response to ER stress has been demonstrated in filamentous fungi. Also, the *S. cerevisiae* genome seems to lack a PERK-encoding gene (Ma and Hendershot, 2001). The transcriptional down-regulation effect found in *A. niger* may be a different method to solve the same problem, which is to limit the entry of nascent protein chains into the ER under conditions when such proteins would be unlikely to fold correctly.

Gene expression can be controlled by DNA sequence elements that may be found at relatively large bp distances from the initiation ATG codon. Gene regulatory proteins can either increase (inducers) or decrease (repressors) the activity of RNA polymerase bound to the promoter. In eukaryotes, transcription enhancers are DNA sequences that, after binding of activator proteins, can activate transcription over distances as large as 80 kb (Jack *et al.*, 1991). Enhancer activity is typically independent of orientation and could be located either up-stream or down-stream of the transcriptional unit it controls (Bondarenko *et al.*,

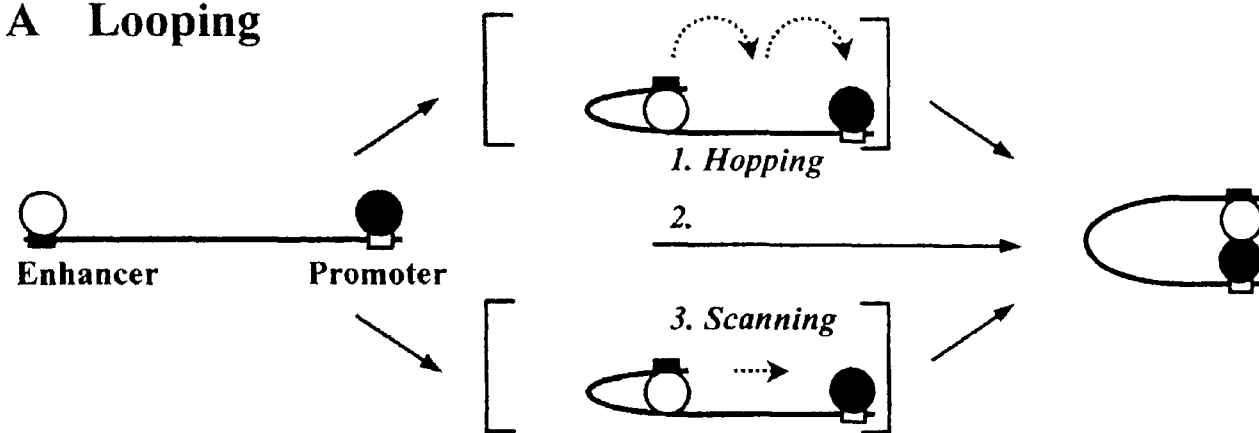
2003). In contrast, in yeast, the upstream activation sites (UASs), enhancer-like elements, mainly work over a short distance (from about 0.1 to 1.4 kb) and, unlike enhancers, they are typically required for gene expression (Guarente, 1987; Struhl, 1989).

Several models have been proposed to explain enhancer action over a distance. Some, but not all, of these models are illustrated in Figure 6.1 and include DNA looping, tracking and spreading/looping (Bondarenko *et al.*, 2003). All models assume that activators work through a protein-protein interaction with a component of the transcription machinery. Briefly, in the DNA looping model, interaction between the enhancer-bound activator with a protein at the promoter is facilitated by looping of the intervening DNA (Ptashne, 1986). Three ways have been proposed for the enhancer-bound activator to find the promoter in this model. First, the enhancer-bound activator can scan surrounding DNA regions using “hopping” or “scanning” mechanisms (Figure 6.1 A (1) and (3), respectively). Alternatively, the enhancer-bound activator might interact directly with the promoter (Figure 6.1 2). However, “hopping” or “scanning” mechanisms may greatly facilitate communication between the promoter and the enhancer (Bondarenko *et al.*, 2003). One of the often-studied eukaryotic enhancers that may demonstrate the DNA looping model is the mammalian β -globin locus control region (LCR) (Li *et al.*, 2002). The second proposed method of enhancer-promoter communication is the tracking model. In this model the enhancer is said to behave like a loading platform for a DNA-tracking protein. At the present time, there is no conclusive evidence for this model in eukaryotes, but a good example of this model can be seen in the case of the transcriptional activation of the bacteriophage T4 late genes (Kolesky *et al.*, 2002). The third proposed model for enhancer-promoter communication is the spreading-looping model. This model suggests that binding of an activator to the enhancer induces cooperative binding-polymerization of a protein on the DNA. A series of relatively small DNA loops is formed during polymerization of an activator, and eventually these small loops bring the enhancer and the promoter into physical proximity (Figure 1 C) (Bondarenko *et al.*, 2003; Dorsett, 1999). A good example is the case of

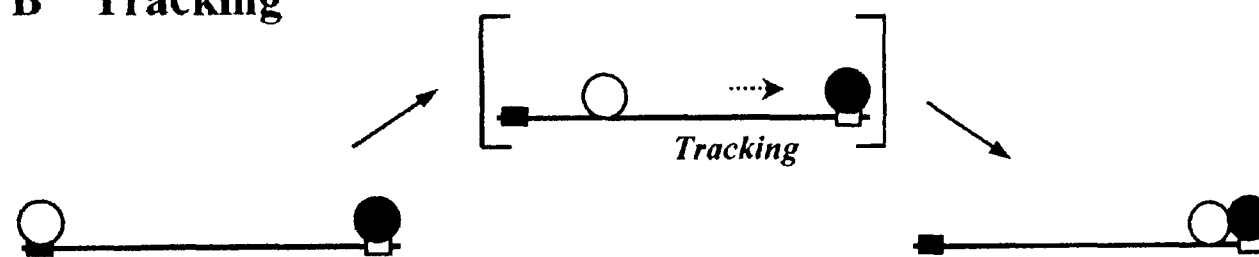
Drosophila CHIP protein, an enhancer-facilitator that helps form chromatin structures that bring the enhancer and promoter closer together (Torigoi *et al.*, 2000). The above proposed models demonstrate some, but not all, methods for enhancer-promoter communication over a large distance. A question that then arises is how can the enhancer be regulated? This may occur through the use of insulators, which are specific DNA sequences that, after binding to corresponding proteins, can limit enhancer action. Protein insulator complexes may have two primary functional activities: 1- position-dependent enhancer blocking (requires the presence of the insulator between the promoter and the enhancer) (Chung *et al.*, 1993), 2- position-independent enhancer blocking by forming chromatin boundaries (Ishii *et al.*, 2002). In these ways, the genomic region, flanked by insulators, behaves as a functionally-independent unit of gene regulation, isolated from both negative and positive effects of surrounding genome regions (Bondarenko *et al.*, 2003). It has been shown that two insulators flanking an enhancer or promoter are required for full repression of stable transgenes (Farrell *et al.*, 2002), and it has been suggested that insulators might functionally interact forming a chromatin loop (Bondarenko *et al.*, 2003). Other experiments, *in vivo*, also suggest pairwise interaction between insulators (Cai and Sin 2001, Murovyova 2001).

Figure 6.1 Models proposed to explain enhancer-promoter communication over a large distance. Enhancer- and promoter-bound proteins are indicated by circles (white and black, respectively) and their DNA-binding sites are indicated by rectangles. The intermediate complexes formed during the enhancer-promoter communication are indicated in brackets. (A) Looping model: enhancer-bound proteins directly interact with a protein at the promoter (2), or search the spacer DNA using hopping (1) or scanning (3). (B) Tracking model and (C) Spreading-looping model. The Spreading-looping model (C) is facilitated by first binding the activator to the enhancer, which induces the binding of another protein (it could be the enhancer-binding protein or another activator) (grey circles) that could be stabilized by a non-DNA-binding protein (white ovals). Figure taken from (Bondarenko *et al.*, 2003).

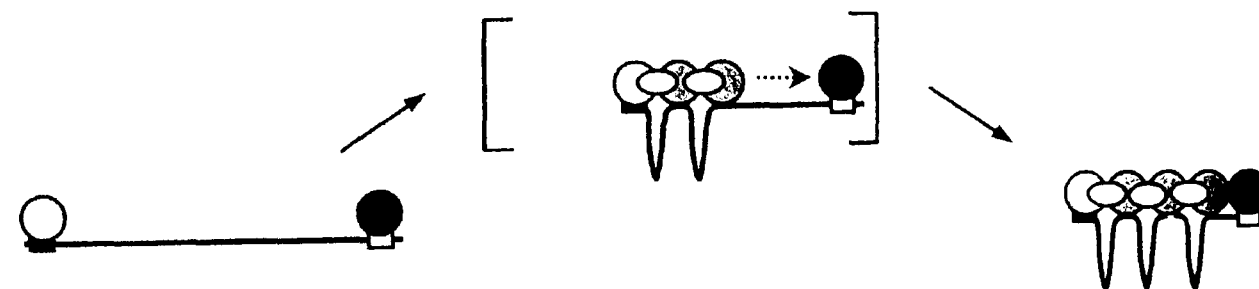
A Looping



B Tracking



C Spreading/looping



In my studies, the difference in the levels of *uidA* expression between the *A. niger* GUS64 (2.032 kb P_{glaA}) and GUS64*SalI* (1.195 kb P_{glaA}) or other *A. niger* reporter strains (Table 4.2) suggests the presence of a regulatory region in the P_{glaA} lying 1.192-2.032 kb upstream of the translational start. However, more studies, including EMSA and DNase I footprinting, are required to further investigate not only the possibility of the presence of an enhancer, but to pinpoint the area of the promoter which is important in mediating the effect and elucidating the mechanism by which the P_{glaA} activity is reduced under ER-stress.

Another question raised in this study is whether or not the transcriptional down-regulation mechanism is mediated separately from the UPR. Several observations suggest that the two mechanisms are controlled differently. First, the antisense *pdiA* strain of *A. niger* (AS1.1) (Ngiam *et al.*, 2000) showed a down-regulation of *glaA*, but the UPR was not evident (Chapter 3). Although there was a difference in the timescale for detecting the decline in *glaA* transcripts during the DTT-stress experiment and in the AS1.1 strain, this difference in the timescale was unavoidable given the difference between a chemical stress agent and an induced response in a strain altered by genetic manipulation. More importantly, the correct controls for each set of experiments were included. Initial results generated from micro-array experiments performed on *A. niger* showed that tunicamycin treatment did not result in down-regulation of genes encoding secreted proteins, whereas DTT treatment did result in down-regulation of several (but not all) genes encoding secreted proteins (Guillemette, T. University of Nottingham, personal communication). It has not, until very recently (H. Mulder, unpublished data), been possible to produce strains of *A. niger* which are deleted for *hacA*, to facilitate investigation of the suggestion that UPR and transcriptional down-regulation are controlled differently. This strain is very sick and it is not known how useful it will prove to be. Moreover, no *A. niger* strain that is deleted for *ireA* is available yet.

In Chapter 3 it was shown that tunicamycin is a poor ER-stress agent compared to DTT as investigated by *hacA* mRNA truncation and intron splicing in

A. niger. Several recent studies agree with this observation. More specifically, in *T. reesei* it was noticed that tunicamycin poorly activated the UPR (Pakula *et al.*, 2003). Also, in *A. niger*, tunicamycin was shown to be not as strong as DTT. In one study tunicamycin showed variable results and did not cause splicing of *hacA* mRNA each time (Mulder, personal communication). Also, using microarray studies, treatment of *A. niger* with tunicamycin did not result in down-regulation of genes encoding secreted proteins (Guillemette, T, University of Nottingham, personal communication). Although using tunicamycin as an ER-stress agent with a medium-exchange prior to adding it to *A. niger* cultures results in more *hacA* mRNA intron splicing (as detected by RT-PCR), it was obvious that tunicamycin is not as strong as DTT as an ER-stress agent (Chapter 3). Thus, tunicamycin was not favoured to be used as an ER-stress agent in this study. Finally, the age of the fungal cultures (44 hours) at the time of adding the tunicamycin may play a role in conferring resistance of the *A. niger* cultures to the tunicamycin, thus, it might be better to add the tunicamycin to younger cultures (e.g. 12 hours).

Although expression of some heterologous proteins has been shown to activate the UPR in fungi (Mulder *et al.*, 2004; Pakula *et al.*, 2001), the UPR was not activated in *A. niger* B1 strain overexpresses HEWL protein in ACMS/N/P medium. However, the expression of heterologous proteins may not induce a clear UPR stress all the time. For example, the *bipA* level, which is induced when UPR is activated, remained unchanged when the heterologous gene encoding interleukin-6 was expressed in *A. niger* (Punt *et al.*, 1998). Thus, the observation that the expression of some heterologous protein may not activate the UPR in *A. niger* could indirectly indicate the high efficiency of *A. niger* as a host for the production of non-native proteins.

The observation that genes encoding secreted proteins or genes encoding proteins targeted to the cell wall are down-regulated under DTT stress in *A. niger* (Guillemette, T, University of Nottingham, personal communication), *T. reesei* (Pakula *et al.*, 2003) and *A. thaliana* (Martinez and Chrispeels, 2003), respectively, may suggest the possibility that at least some of these genes may be regulated by transcriptional down-regulation mechanism. If this is true, this may suggest a

presence of a conserved element(s) that may be responsible for mediating the transcriptional down-regulation of these genes. To investigate this observation in more detail, the region(s) in the P_{glaA} needs to be narrowed down, possibly by EMSA and DNase I footprinting analysis. Once this region(s) is identified, it might be possible to selectively regulate the target gene expressed under the control of P_{glaA} . This could be achieved by genetically modifying P_{glaA} to alter its responsiveness to the transcriptional down-regulation. Furthermore, identifying the target sequence(s) in P_{glaA} may assists in identifying the transcriptional factor(s) that is possibly involved in mediating the transcriptional down-regulation. Thus, by identifying the gene(s) coding for regulatory factors mediating the transcriptional down-regulation it might be possible to genetically modify the fungal strain so that the transcriptional down-regulation effect is either abolished or enhanced. Inactivation of the transcriptional down-regulation mechanism might be desired when the production of the target gene is under the control of modified P_{glaA} that is not subjected to transcriptional down-regulation. On the other hand, enhancement of transcriptional down-regulation might be achieved to repress the production of undesired proteins, expressed under the control of promoters that are transcriptionally down-regulated, when the expression of the protein of interest under a promoter is not subjected to transcriptional down-regulation. After identifying the target sequence(s), the P_{glaA} can be modified by DNA techniques such as site directed mutagenesis or deletion.

In conclusion, this study describes a new ER-associated stress response in the filamentous fungus *A. niger*, termed transcriptional down-regulation under secretion stress. This mechanism was activated by treating *A. niger* cultures with DTT, which causes the formation of unfolded proteins. DTT down-regulated the transcription of the gene encoding *glaA*, a major secreted protein, but not the gene encoding the non-secreted protein, actin. The transcriptional down-regulation effect is shown by nuclear run-on studies to be at the level of transcription, rather than mRNA stability, and is found to be mediated through the P_{glaA} in a region more than 1kb upstream of the translational start. The DTT-treated fungal cells also showed evidence of the induction of the UPR, because expression of *bipA*

was up-regulated and splicing of *hacA* mRNA occurred. Furthermore, preliminary results using the *pdiA* antisense strain, where the steady-state *glaA* mRNA level was shown previously to be lowered, suggest that the transcriptional down-regulation mechanism is controlled differently to the UPR, since there was no evidence of UPR activation in the *pdiA* antisense strain.

Finally, the UPR, ERAD and transcriptional down-regulation mechanisms may help the cell to cope with ER-stress by improving protein folding and transport, degrading unwanted proteins, and allowing fewer secretory proteins to enter the ER, respectively.

References

- Abcede, R. A. N. and W. L. Barraquio. (2003) Factors influencing the expression of *bglA* gene in *Aspergillus kawachii* (Kitahara and Yoshida) sC(-)strain. *Philipp. Agric. Sci.* **86**, 377-384.
- Alberini, C., P. Bet, C. Milstein and R. Sitia. (1990) Secretion of immunoglobulin-M assembly intermediates in the presence of reducing agents. *Nature* **347**, 485-487.
- Alcocer, M. J. C., C. S. M. Furniss, P. A. Kroon, M. Campbell and D. B. Archer. (2003) Comparison of modular and non-modular xylanases as carrier proteins for the efficient secretion of heterologous proteins from *Penicillium funiculosum*. *Appl. Microbiol. Biotechnol.* **60**, 726-732.
- Al-Sheikh, H., A. J. Watson, G. A. Lacey, P. J. Punt, D. A. MacKenzie, D. J. Jeenes, T. Pakula, M. Penttilä, M. J. C. Alcocer and D. B. Archer. (2004) Endoplasmic reticulum stress leads to the selective transcriptional downregulation of the glucoamylase gene in *Aspergillus niger*. *Mol. Microbiol.* **53**, 1731-1742.
- Anderson, J. S. J. and R. Parker. (1998) The 3' to 5' degradation of yeast mRNAs is a general mechanism for mRNA turnover that requires the SKI2 DEVH box protein and 3' to 5' exonucleases of the exosome complex. *EMBO J.* **17**, 1497-1506.
- Andrianopoulos, A. and M. J. Hynes. (1988) Cloning and analysis of the positively acting regulatory gene *amdR* from *Aspergillus nidulans*. *Mol. Cell. Biol.* **8**, 3532-3541.
- Anke, T. (1997) Fungal biotechnology. London, Chapman and Hall.
- Archer, D. B. (2000) Filamentous fungi as microbial cell factories for food use. *Curr. Opin. Biotechnol.* **11**, 478-483.
- Archer, D. B. and P. S. Dyer. (2004) From genomics to post-genomics in *Aspergillus*. *Curr. Opin. Microbiol.* **7**, 499-504.

Archer, D. B., D. J. Jeenes and D. A. MacKenzie. (1994a) Strategies for improving heterologous protein production from filamentous fungi. *Antonie Van Leeuwenhoek* **65**, 245-250.

Archer, D. B., D. J. Jeenes, D. A. MacKenzie, G. Brightwell, N. Lambert, G. Lowe, S. E. Radford and C. M. Dobson. (1990a) Hen egg-white lysozyme expressed in, and secreted from, *Aspergillus niger* is correctly processed and folded. *Biotechnology* **8**, 741-745.

Archer, D. B., D. A. MacKenzie, D. J. Jeenes and I. N. Roberts. (1992) Proteolytic degradation of heterologous proteins expressed in *Aspergillus niger*. *Biotechnol. Lett.* **14**, 357-362.

Archer, D. B. and J. F. Peberdy. (1997) The molecular biology of secreted enzyme production by fungi. *Crit. Rev. Biotechnol.* **17**, 273-306.

Aridor, M. and W. E. Balch. (1999) Integration of endoplasmic reticulum signaling in health and disease. *Nat. Med.* **5**, 745-751.

Arima, H., T. Kinoshita, H. R. Ibrahim, H. Azakami and A. Kato. (1998) Enhanced secretion of hydrophobic peptide fused lysozyme by the introduction of N-glycosylation signal and the disruption of calnexin gene in *Saccharomyces cerevisiae*. *FEBS Lett.* **440**, 89-92.

Barz, W. P. and P. Walter. (1999) Two endoplasmic reticulum (ER) membrane proteins that facilitate ER to Golgi transport of glycosylphosphatidylinositol-anchored proteins. *Mol. Biol. Cell* **10**, 1043-1059.

Benedetti, C., M. Fabbri, R. Sitia and A. Cabibbo. (2000) Aspects of gene regulation during the UPR in human cells. *Biochem. Biophys. Res. Commun.* **278**, 530-536.

Beri, R. K., S. Grant, C. F. Roberts, M. Smith and A. R. Hawkins. (1990) Selective overexpression of the *qute* gene encoding catabolic 3- dehydroquinase in multicopy transformants of *Aspergillus nidulans*. *Biochem. J.* **265**, 337-342.

Berka, R. M., M. Ward, L. J. Wilson, K. J. Hayenga, K. H. Kodama, L. P. Carlomagno and S. A. Thompson. (1990) Molecular cloning and deletion of the gene encoding Aspergillopepsin A from *Aspergillus awamori*. *Gene* **86**, 153-162.

Bertolotti, A., X. Z. Wang, I. Novoa, R. Jungreis, K. Schlessinger, J. H. Cho, A. B. West and D. Ron. (2001) Increased sensitivity to dextran sodium sulfate colitis in IRE1 β -deficient mice. *J. Clin. Invest.* **107**, 585-593.

Bertolotti, A., Y. H. Zhang, L. M. Hendershot, H. P. Harding and D. Ron. (2000) Dynamic interaction of BiP and ER stress transducers in the unfolded protein response. *Nat. Cell Biol.* **2**, 326-332.

Bichet, D., V. Cornet, S. Geib, E. Carlier, S. Volsen, T. Hoshi, Y. Mori and M. De Waard. (2000) The I-II loop of the Ca^{2+} channel $\alpha 1$ subunit contains an endoplasmic reticulum retention signal antagonized by the β subunit. *Neuron* **25**, 177-190.

Bird, P., M. Gething and J. Sambrook. (1990) The functional efficiency of a mammalian signal peptide is directly related to its hydrophobicity. *J. Biol. Chem.* **265**, 8420-8425.

Boel, E., M. T. Hansen, I. Hjort, I. Hoegh and N. P. Fiil. (1984a) Two different types of intervening sequences in the glucoamylase gene from *Aspergillus niger*. *EMBO J.* **3**, 1581-1585.

Bondarenko, V. A., Y. V. Liu, Y. I. Jiang and V. M. Studitsky. (2003) Communication over a large distance: enhancers and insulators. *Biochem. Cell Biol.* **81**, 241-251.

Braakman, I., J. Helenius and A. Helenius. (1992) Manipulating disulfide bond formation and protein folding in the endoplasmic reticulum. *EMBO J.* **11**, 1717-1722.

Brakhage, A., A. Andrianopoulos, M. Kato, S. Steidl, M. A. Davis, N. Tsukagoshi and M. J. Hynes. (1999) HAP-like CCAAT-binding complexes in filamentous fungi: Implications for biotechnology. *Fungal Genet. Biol.* **27**, 243-252.

Brewer, J. W. and J. A. Diehl. (2000) PERK mediates cell-cycle exit during the mammalian unfolded protein response. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 12625-12630.

Broekhuijsen, M. P., I. E. Mattern, R. Contreras, J. R. Kinghorn and C. A. M. J. J. van den Hondel. (1993) Secretion of heterologous proteins by *Aspergillus niger* production of active human interleukin-6 in a protease- deficient mutant by Kex2-like processing of a glucoamylase-Hil6 fusion protein. *J. Biotechnol.* **31**, 135-145.

- Brostrom, C. O., K. V. Chin, W. L. Wong, C. Cade and M. A. Brostrom. (1989) Inhibition of translational initiation in eukaryotic cells by calcium ionophore. *J. Biol. Chem.* **264**, 1644-1649.
- Brown, R. L., C. S. Brown-Jenco, D. Bhatnagar and G. A. Payne. (2003) Construction and preliminary evaluation of an *Aspergillus flavus* reporter gene construct as a potential tool for screening aflatoxin resistance. *J. Food Prot.* **66**, 1927-1931.
- Bu, G. J., H. J. Geuze, G. J. Strous and A. L. Schwartz. (1995) 39-kDa Receptor-associated protein is an ER resident protein and molecular chaperone for Ldl receptor-related protein. *EMBO J.* **14**, 2269-2280.
- Calton, M., H. Q. Zeng, F. Urano, J. H. Till, S. R. Hubbard, H. P. Harding, S. G. Clark and D. Ron. (2002) IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature* **415**, 92-96.
- Canet, D., A. M. Last, P. Tito, M. Sunde, A. Spencer, D. B. Archer, C. Redfield, C. V. Robinson and C. M. Dobson. (2002) Local cooperativity in the unfolding of an amyloidogenic variant of human lysozyme. *Nat. Struct. Biol.* **9**, 308-315.
- Cary, J. W., B. G. Montalbano and K. C. Ehrlich. (2000) Promoter elements involved in the expression of the *Aspergillus parasiticus* aflatoxin biosynthesis pathway gene *avnA*. *Biochim. Biophys. Acta-Gene Struct. Expression* **1491**, 7-12.
- Casagrande, R., P. Stern, M. Diehn, C. Shamu, M. Osario, M. Zuniga, P. O. Brown and H. Ploegh. (2000) Degradation of proteins from the ER of *Saccharomyces cerevisiae* requires an intact unfolded protein response pathway. *Mol. Cell.* **5**, 729-735.
- Chapman, R., C. Sidrauski and P. Walter. (1998) Intracellular signaling from the endoplasmic reticulum to the nucleus. *Annu. Rev. Cell Dev. Biol.* **14**, 459-485.
- Chaudhuri, M. M., P. N. Tonin, W. H. Lewis and P. R. Srinivasan. (1992) The gene for a novel protein, a member of the protein disulfide isomerase/form-I phosphoinositide-specific phospholipase-C family, is amplified in hydroxyurea-resistant cells. *Biochem. J.* **281**, 645-650.
- Chen, H. X., J. W. Crabb and J. A. Kinsey. (1998) The *Neurospora aab1* gene encodes a CCAAT binding protein homologous to yeast HAP5. *Genetics* **148**, 123-130.

- Chen, H. X. and J. A. Kinsey. (1995) Purification of a heteromeric CCAAT binding-protein from *neurospora crassa*. *Mol. Gen. Genet.* **249**, 301-308.
- Choukhi, A., S. Ung, C. Wychowski and J. Dubuisson. (1998) Involvement of endoplasmic reticulum chaperones in the folding of hepatitis C virus glycoproteins. *J. Virol.* **72**, 3851-3858.
- Chung, J. H., M. Whiteley and G. Felsenfeld. (1993) A 5' Element of the chicken β -globin domain serves as an insulator in human erythroid cells and protects against position effect in *Drosophila*. *Cell* **74**, 505-514.
- Conesa, A., D. Jeenes, D. B. Archer, C. A. M. J. J. van den Hondel and P. J. Punt. (2002) Calnexin overexpression increases manganese peroxidase production in *Aspergillus niger*. *Appl. Environ. Microbiol.* **68**, 846-851.
- Conesa, A., P. J. Punt, N. van Luijk and van den Hondel, C.A.M.J.J. (2001a) The secretion pathway in filamentous fungi: A biotechnological view. *Fungal Genet. Biol.* **33**, 155-171.
- Conesa, A., C.A.M.J.J. van den Hondel and P. J. Punt. (2000) Studies on the production of fungal peroxidases in *Aspergillus niger*. *Appl. Environ. Microbiol.* **66**, 3016-3023.
- Conesa, A., G. Weelink, C.A.M.J.J. van den Hondel and P. J. Punt. (2001b) C-terminal propeptide of the *Caldariomyces fumago* chloroperoxidase: an intramolecular chaperone? *FEBS Lett.* **503**, 117-120.
- Contreras, R., D. Carrez, J. R. Kinghorn, C.A.M.J.J. van den Hondel and W. Fiers. (1991) Efficient Kex2-like processing of a glucoamylase-interleukin-6 fusion protein by *Aspergillus nidulans* and secretion of mature interleukin-6. *Biotechnology* **9**, 378-381.
- Cox, J., R. Chapman and P. Walter. (1997) The unfolded protein response coordinates the production of endoplasmic reticulum protein and endoplasmic reticulum membrane. *Mol. Biol. Cell* **8**, 1805-1814.
- Cox, J., C. E. Shamu and P. Walter. (1993) Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase. *Cell* **73**, 1197-1206.

- Cox, J. and P. Walter. (1996) A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response. *Cell* **87**, 391-404.
- Cyr, D., X. Lu and M. Douglas. (1992) Regulation of Hsp70 function by a eukaryotic DnaJ homolog. *J. Biol. Chem.* **267**, 20927-20931.
- Davies, W. R., Ed. (1994). Heterologous gene expression and protein secretion in *Aspergillus*. Progress in industrial microbiology. Amsterdam, Elsevier.
- de Faria, F. P., V. S. J. Te'o, P. L. Bergquist, M. O. Azevedo and K. M. H. Nevalainen. (2002) Expression and processing of a major xylanase (XYN2) from the thermophilic fungus *Humicola grisea* var. *thermoidea* in *Trichoderma reesei*. *Let. Appl. Microbiol.* **34**, 119-123.
- Deshaies, R. J., B. D. Koch, M. Wernerwashburne, E. A. Craig and R. Schekman. (1988) A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature* **332**, 800-805.
- Dikshit, K. L. and D. A. Webster. (1988) Cloning, characterization and expression of the bacterial globin gene from vitreoscilla in *Escherichia coli*. *Gene* **70**, 377-386.
- Dorner, A. J., L. C. Wasley, P. Raney, S. Haugejorden, M. Green and R. J. Kaufman. (1990) The stress response in chinese hamster ovary cells regulation of Erp72 and protein disulfide isomerase expression and secretion. *J. Biol. Chem.* **265**, 22029-22034.
- Dorsett, D. (1999) Distant liaisons: long-range enhancer-promoter interactions in *Drosophila*. *Curr. Opin. Genet. Dev.* **9**, 505-514.
- Drysdale, M. R., S. E. Kolze and J. M. Kelly. (1993) The *Aspergillus niger* carbon catabolite repressor encoding gene, *creA*. *Gene* **130**, 241-245.
- Durand, H., M. Clanet and G. Tiraby. (1988) Genetic improvement of *Trichoderma reesei* for large scale cellulase production. *Enz. Microb. Technol.* **10**, 341-346.
- Ellgaard, L. and A. Helenius. (2003) Quality control in the endoplasmic reticulum. *Nat. Rev. Mol. Cell Biol.* **4**, 181-191.
- Ellgaard, L., M. Molinari and A. Helenius. (1999) Setting the standards: quality control in the secretory pathway. *Science* **286**, 1882-1888.

- Ellis, R. J. (1993) The general concept of molecular chaperones. *Phil. Trans. R. Soc. Lond. Ser. B-Biol. Sci.* **339**, 257-261.
- Fagioli, C. and R. Sitia. (2001) Glycoprotein quality control in the endoplasmic reticulum. Mannose trimming by endoplasmic reticulum mannosidase I times the proteasomal degradation of unassembled immunoglobulin subunits. *J. Biol. Chem.* **276**, 12885-12892.
- Fan, X. C., R. X. Qiu, L. Liu and G. M. Tang. (2001) Study on the molecular basis of glucoamylase overproduction of a mutant strain *Aspergillus niger* T21. *Sci. China Ser. C-Life Sci.* **44**, 287-293.
- Farrell, C. M., A. G. West and G. Felsenfeld. (2002) Conserved CTCF insulator elements flank the mouse and human β -globin loci. *Mol. Cell. Biol.* **22**, 3820-3831.
- Farrell, R. E. (1997) *RNA Methodologies: A laboratory guide for isolation and characterization*. New York, Academic Press.
- Fernandez, J., I. Yaman, W. C. Merrick, A. Koromilas, R. C. Wek, R. Sood, J. Hensold and M. Hatzoglou. (2002) Regulation of internal ribosome entry site-mediated translation by eukaryotic initiation factor-2 α -phosphorylation and translation of a small upstream open reading frame. *J. Biol. Chem.* **277**, 2050-2058.
- Fidel, S., J. H. Doonan and N. R. Morris. (1988) *Aspergillus nidulans* contains a single actin gene which has unique intron locations and encodes a gamma actin. *Gene* **70**, 283-293.
- Finkelstein, D. B., J. Rambosek, M. S. Crawford, C. L. Soliday, P. C. McAda, and J. Leach. (1989) Protein secretion in *Aspergillus niger*. In C. L. Hershberger, S. W. Queener, and G. Hegeman (ed.), *Genetics and molecular biology of industrial microorganisms*. American Society for Microbiology, Washington, D.C. p. 295-300
- Flaus, A., C. Rencurel, H. Ferreira, N. Wiechens and T. Owen-Hughes. (2004) *S/N1* mutations alter inherent nucleosome mobility. *EMBO J.* **23**, 343-353.
- Flynn, G. C., J. Pohl, M. T. Flocco and J. E. Rothman. (1991) Peptide binding specificity of the molecular chaperone Bip. *Nature* **353**, 726-730.
- Fowler, T., R. M. Berka and M. Ward. (1990) Regulation of the *glaA* gene of *Aspergillus niger*. *Curr. Genet.* **18**, 537-545.

Frand, A. R. and C. A. Kaiser. (1998) The *ERO1* gene of yeast is required for oxidation of protein dithiols in the endoplasmic reticulum. *Mol. Cell.* **1**, 161-170.

Frederick, G. D. and J. A. Kinsey. (1990) Distant upstream regulatory sequences control the level of expression of the *Am (gdh)* locus of *Neurospora crassa*. *Curr. Genet.* **18**, 53-58.

Freeman, S., M. Maymon, B. Kirshner, D. Rav-David and Y. Elad. (2002) Use of GUS transformants of *Trichoderma harzianum* isolate T39 (TRICHODEX) for studying interactions on leaf surfaces. *Biocontrol Sci. Technol.* **12**, 401-407.

Gansheroff, L. J., C. Dollard, P. Tan and F. Winston. (1995) The *Saccharomyces cerevisiae* SPT7 gene encodes a very acidic protein important for transcription *In vivo*. *Genetics* **139**, 523-536.

Goller, S. P., M. Gorfer and C. P. Kubicek. (1998) *Trichoderma reesei* *prs12* encodes a stress- and unfolded-protein response inducible regulatory subunit of the fungal 26S proteasome. *Curr. Genet.* **33**, 284-290.

Gomi, K., T. Akeno, T. Minetoki, K. Ozeki, C. Kumagai, N. Okazaki and Y. Imura. (2000) Molecular cloning and characterization of a transcriptional activator gene, *amyR*, involved in the amylolytic gene expression in *Aspergillus oryzae*. *Biosci. Biotechnol. Biochem.* **64**, 816-827.

Gorlich, D., E. Hartmann, S. Prehn and T. A. Rapoport. (1992a) A protein of the endoplasmic reticulum involved early in polypeptide translocation. *Nature* **357**, 47-52.

Gorlich, D., S. Prehn, E. Hartmann, K. U. Kalies and T. A. Rapoport. (1992a) A mammalian homolog of Sec61p and Secyp is associated with ribosomes and nascent polypeptides during translocation. *Cell* **71**, 489-503.

Gorlich, D. and T. A. Rapoport. (1993) Protein translocation into proteoliposomes reconstituted from purified components of the endoplasmic reticulum membrane. *Cell* **75**, 615-630.

Gouka, R. J., P. J. Punt, J. G. M. Hessing and van den Hondel, C.A.M.J.J. (1996a) Analysis of heterologous protein production in defined recombinant *Aspergillus awamori* strains. *Appl. Environ. Microbiol.* **62**, 1951-1957.

Gouka, R. J., P. J. Punt and C. A. M. J. J. van den Hondel. (1997a) Efficient production of secreted proteins by *Aspergillus*: progress, limitations and prospects. *Appl. Microbiol. Biotechnol.* **47**, 1-11.

Gouka, R. J., P. J. Punt and C. A. M. J. J. van den Hondel. (1997b) Glucoamylase gene fusions alleviate limitations for protein production in *Aspergillus awamori* at the transcriptional and (post)translational levels. *Appl. Environ. Microbiol.* **63**, 488-497.

Grant, P., L. Duggan, J. Cote, S. Roberts, J. Brownell, R. Candau, R. Ohba, T. Owen- Hughes, C. Allis, F. Winston, S. Berger and J. Workman. (1997) Yeast Gcn5p functions in two multisubunit complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA (Spt/Ada) complex. *Genes Dev.* **11**, 1640-1650.

Groot, G., H. Pel, N. van. Peij and A. van. Ooyen.(2002) Exploring and exploiting the *Aspergillus niger* genome, SIM annual meeting abstracts S68: 77.

Guarente, L. (1987) Regulatory Proteins in Yeast. *Annu. Rev. Genet.* **21**, 425-452.

Guhaniyogi, J. and G. Brewer. (2001) Regulation of mRNA stability in mammalian cells. *Gene* **265**, 11-23.

Hai, T. W., F. Liu, W. J. Coukos and M. R. Green. (1989) Transcription factor Atf cDNA clones - an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. *Genes Dev.* **3**, 2083-2090.

Hamilton, T. G. and G. C. Flynn. (1996) Cer1p, a novel Hsp70-related protein required for posttranslational endoplasmic reticulum translocation in yeast. *J. Biol. Chem.* **271**, 30610-30613.

Harding, H. P., I. Novoa, Y. H. Zhang, H. Q. Zeng, R. Wek, M. Schapira and D. Ron. (2000) Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol. Cell.* **6**, 1099-1108.

Harding, H. P., Y. H. Zhang and D. Ron. (1999) Protein translation and folding are coupled by an endoplasmic reticulum-resident kinase. *Nature* **397**, 271-274.

Harter, C. and F. Wieland. (1996) The secretory pathway: mechanisms of protein sorting and transport. *Biochim. Biophys. Acta-Rev. Biomembr.* **1286**, 75-93.

Hartmann, E., T. Sommer, S. Prehn, D. Gorlich, S. Jentsch and T. A. Rapoport. (1994) Evolutionary conservation of components of the protein translocation complex. *Nature* **367**, 654-657.

Hata, Y. and H. Ishida. (2000) Glucoamylase-encoding genes of *Aspergillus oryzae* Monograph. *Seibutsu-Kogaku Kaishi-J. Soc. Ferment. Bioeng.* **78**, 120-127.

Hata, Y., H. Ishida, E. Ichikawa, A. Kawato, K. Suginami and S. Imayasu. (1998) Nucleotide sequence of an alternative glucoamylase-encoding gene (*glbB*) expressed in solid-state culture of *Aspergillus oryzae*. *Gene* **207**, 127-134.

Haze, K., H. Yoshida, H. Yanagi, T. Yura and K. Mori. (1999) Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. *Mol. Biol. Cell* **10**, 3787-3799.

Heikkinen, H. L., S. A. Llewellyn and C. A. Barnes. (2003) Initiation-mediated mRNA decay in yeast affects heat-shock mRNAs, and works through decapping and 5'-to-3' hydrolysis. *Nucleic Acids Res.* **31**, 4006-4016.

Helenius, A., T. Marquardt and I. Braakman. (1992) The endoplasmic reticulum as a protein-folding compartment. *Trends in Cell Biology* **2**, 227-231.

Helenius, A., E. S. Trombetta, D. N. Hebert and J. F. Simons. (1997) Calnexin, calreticulin and the folding of glycoproteins. *Trends in Cell Biology* **7**, 193-200.

Hellman, R., M. van hove, A. Lejeune, F. J. Stevens and L. M. Hendershot. (1999) The *in vivo* association of BiP with newly synthesized proteins is dependent on the rate and stability of folding and not simply on the presence of sequences that can bind to BiP. *J. Cell Biol.* **144**, 21-30.

Henriksen, A. L. S., S. Even, C. Muller, P. J. Punt, C. A. M. J. J. van den Hondel and J. Nielsen. (1999) Study of the glucoamylase promoter in *Aspergillus niger* using green fluorescent protein. *Microbiology-(UK)* **145**, 729-734.

Herrmann, J. M., P. Malkus and R. Schekman. (1999) Out of the ER - outfitters, escorts and guides. *Trends in Cell Biology* **9**, 5-7.

Hessing, J., L. G. Frenken, G. M. van Muijlwijk-Harteveld, W. Musters and C. A. M. J. J. van den Hondel. (1996) Studies on the production of single-chain antibody fragments by *Aspergillus*. *Fungal Genetics Newsletter*. 43B 23.

Hori, O., F. Ichinoda, A. Yamaguchi, T. Tamatani, M. Taniguchi, Y. Koyama, T. Katayama, M. Tohyama, D. M. Stern, K. Ozawa, Y. Kitao and S. Ogawa. (2004) Role of Herp in the endoplasmic reticulum stress response. *Genes Cells* **9**, 457-469.

Hubbard, S. C. and R. J. Ivatt. (1981) Synthesis and processing of asparagine linked oligosaccharides. *Annu. Rev. Biochem.* **50**, 555-583.

Ilmén, M., M. L. Onnela, S. Klemsdal, S. Keranen and M. Penttilä. (1996) Functional analysis of the cellobiohydrolase I promoter of the filamentous fungus *Trichoderma reesei*. *Mol. Gen. Genet.* **253**, 303-314.

Ilmén, M., A. Saloheimo, M. L. Onnela and M. E. Penttilä. (1997) Regulation of cellulase gene expression in the filamentous fungus *Trichoderma reesei*. *Appl. Environ. Microbiol.* **63**, 1298-1306.

Ishida, H., Y. Hata, A. Kawato, Y. Abe, K. Suginami and S. Imayasu. (2000) Identification of functional elements that regulate the glucoamylase-encoding gene (*glaB*) expressed in solid-state culture of *Aspergillus oryzae*. *Curr. Genet.* **37**, 373-379.

Ishii, K., G. Arib, C. Lin, G. van Houwe and U. K. Laemmli. (2002) Chromatin boundaries in budding yeast: The nuclear pore connection. *Cell* **109**, 551-562.

Isono, E., Y. Saeki, H. Yokosawa and A. Toh. (2004) RPN7 is required for the structural integrity of the 26S proteasome of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **279**, 27168-27176.

Jack, J., D. Dorsett, Y. Delotto and S. Liu. (1991) Expression of the cut locus in the *Drosophila* wing margin is required for cell type specification and is regulated by a distant enhancer. *Development* **113**, 735-747.

Jalving, R., P. J. I. van de Vondervoort, J. Visser and P. J. Schaap. (2000) Characterization of the kexin-like maturase of *Aspergillus niger*. *Appl. Environ. Microbiol.* **66**, 363-368.

Jannatipour, M. and L. A. Rokeach. (1995) The *Schizosaccharomyces pombe* homolog of the chaperone calnexin is essential for viability. *J. Biol. Chem.* **270**, 4845-4853.

Jeenes, D. J., D. A. MacKenzie and D. B. Archer. (1994) Transcriptional and posttranscriptional events affect the production of secreted hen egg white lysozyme by *Aspergillus niger*. *Transgenic Research* **3**, 297-303.

Jeenes, D. J., D. A. MacKenzie, I. N. Roberts and D. B. Archer. (1991) Heterologous protein production by filamentous fungi. *Biotechnol. Genet. Eng. Rev.* **9**, 327-367.

Jeenes, D. J., R. Pfaller and D. B. Archer. (1997) Isolation and characterisation of a novel stress-inducible PDI- family gene from *Aspergillus niger*. *Gene* **193**, 151-156.

Jefferson, R. A., T. A. Kavanagh and M. W. Bevan. (1987) Gus fusions β -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**, 3901-3907.

Jiang, Y. W. and D. J. Stillman. (1992) Involvement of the Sin4 global transcriptional regulator in the chromatin structure of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **12**, 4503-4514.

Kabani, M., S. S. Kelley, M. W. Morrow, D. L. Montgomery, R. Sivendran, M. D. Rose, L. M. Gierasch and J. L. Bordsky. (2003) Dependence of endoplasmic reticulum-associated degradation on the peptide binding domain and concentration of BiP. *Mol. Biol. Cell* **14**, 3437-3448.

Kato, M., A. Aoyama, F. Naruse, T. Kobayashi and N. Tsukagoshi. (1997) An *Aspergillus nidulans* nuclear protein, AnCP, involved in enhancement of Taka-amylase A gene expression, binds to the CCAAT-containing *taaG2*, *amdS*, and *gatA* promoters. *Mol. Gen. Genet.* **254**, 119-126.

Kawahara, T., H. Yanagi, T. Yura and K. Mori. (1997) Endoplasmic reticulum stress-induced mRNA splicing permits synthesis of transcription factor Hac1p/Ern4p that activates the unfolded protein response. *Mol. Biol. Cell* **8**, 1845-1862.

Kawahara, T., H. Yanagi, T. Yura and K. Mori. (1998) Unconventional splicing of *HAC1/ERN4* mRNA required for the unfolded protein response. Sequence-specific and non- sequential cleavage of the splice sites. *J. Biol. Chem.* **273**, 1802-1807.

- Kimata, Y., Y. L. Kimata, Y. Shimizu, H. Abe, R. C. Farcasanu, M. Takeuchi, M. D. Rose and K. Kohno. (2003) Genetic evidence for a role of BiP/Kar2p that regulates Ire1 in response to accumulation of unfolded proteins. *Mol. Biol. Cell* **14**, 2559-2569.
- Kimata, Y., D. Oikawa, Y. Shimizu, Y. Ishiwata-Kimata and K. Kohno. (2004) A role for BiP as an adjustor for the endoplasmic reticulum stress-sensing protein Ire1. *J. Cell Biol.* **167**, 445-456.
- Klausner, R. D., J. G. Donaldson and J. Lippincottschwartz. (1992) Brefeldin-A: Insights into the control of membrane traffic and organelle structure. *J. Cell Biol.* **116**, 1071-1080.
- Klich, M. A., E. J. Mullaney and C. B. Daly. (1993) Analysis of intraspecific and interspecific variability of 3 common species in *Aspergillus* section versicolores using DNA Restriction Fragment Length Polymorphisms. *Mycologia* **85**, 852-855.
- Kokame, K., H. Kato and T. Miyata. (2001) Identification of ERSE-II, a new cis-acting element responsible for the ATF6-dependent mammalian Unfolded Protein Response. *J. Biol. Chem.* **276**, 9199-9205.
- Kolar, M., P. J. Punt, C. A. M. J. J. van den Hondel and H. Schwab. (1988) Transformation of *Penicillium chrysogenum* using dominant selection markers and expression of an *Escherichia coli lacZ* fusion gene. *Biol. Chem. Hoppe-Seyler* **369**, 852-852.
- Kolesky, S. E., M. Ouhammouch and E. P. Geiduschek. (2002) The mechanism of transcriptional activation by the topologically DNA-linked sliding clamp of bacteriophage T4. *J. Mol. Biol.* **321**, 767-784.
- Krasevec, N., C. A. M. J. J. van den Hondel and R. Komel. (2000a) Can hTNF- α be successfully produced and secreted in filamentous fungus *Aspergillus niger*? *Pflugers Arch.* **439**, R84-R86.
- Krasevec, N., C. A. M. J. J. van den Hondel and R. Komel. (2000b) Expression of human lymphotoxin- α in *Aspergillus niger*. *Pflugers Arch.* **440**, R83-R85.
- Kuhn, K. M., J. L. DeRisi, P. O. Brown and P. Sarnow. (2001) Global and specific translational regulation in the genomic response of *Saccharomyces cerevisiae* to a rapid transfer from a fermentable to a nonfermentable carbon source. *Mol. Cell. Biol.* **21**, 916-927.

Kulmburg, P., M. Mathieu, C. Dowzer, J. Kelly and B. Felenbok. (1993) Specific Binding-Sites in the *alcR* and *alcA* promoters of the ethanol regulon for the CreA repressor mediating carbon catabolite repression in *Aspergillus nidulans*. *Mol. Microbiol.* **7**, 847-857.

Kumeda, Y. and T. Asao. (2001) Heteroduplex panel analysis, a novel method for genetic identification of *Aspergillus section flavi* strains. *Appl. Environ. Microbiol.* **67**, 4084-4090.

Kusters-van Someren, M.A., H.C.M. Kester, R.A. Samson, and J. Visser. (1990) Variations in pectolytic enzymes of the black Aspergilli: A biochemical and genetic approach. *In* R. A. Samson and J. I. Pitt (eds.), Modern concepts in Penicillium and Aspergillus classification. NATO Advanced Science Institute Series, Series A: Life Sciences. Plenum Press, New York, 185.

Lam, Y. A., T. G. Lawson, M. Velayutham, J. L. Zweier and C. M. Pickart. (2002) A proteasomal ATPase subunit recognizes the polyubiquitin degradation signal. *Nature* **416**, 763-767.

Leclerc, G., G. Leclerc and J. Barredo. (2002) Real-time RT-PCR analysis of mRNA decay: half-life of β -actin mRNA in human leukemia CCRF-CEM and Nalm-6 cell lines. *Cancer Cell Int.* **2**, 1-5.

Lee, A. H., N. N. Iwakoshi and L. H. Glimcher. (2003) XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. *Mol. Cell. Biol.* **23**, 7448-7459.

Lee, K., W. Tirasophon, X. H. Shen, M. Michalak, R. Prywes, T. Okada, H. Yoshida, K. Mori and R. J. Kaufman. (2002b) IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. *Genes Dev.* **16**, 452-466.

Lee, S. (1992) Mammalian stress response: induction of the glucose-regulated protein family. *Curr. Opin. Cell Biol.* **4**, 267-273.

Li, M. Q., P. Baumeister, B. Roy, T. Phan, D. Foti, S. Z. Luo and A. S. Lee. (2000) ATF6 as a transcription activator of the endoplasmic reticulum stress element: Thapsigargin stress-induced changes and synergistic interactions with NF-Y and YY1. *Mol. Cell. Biol.* **20**, 5096-5106.

- Li, Q. L., K. R. Peterson, X. D. Fang and G. Stamatoyannopoulos. (2002) Locus control regions. *Blood* **100**, 3077-3086.
- Li, Y., S. Bjorklund, Y. Jiang, Y. Kim, W. Lane, D. Stillman and R. Kornberg. (1995) Yeast global transcriptional regulators Sin4 and Rgr1 are components of mediator complex/RNA polymerase II holoenzyme. *Proc. Natl. Acad. Sci. USA* **92**, 10864-10868.
- Li, Y., R. D. Moir, I. K. Sethy-Coraci, J. R. Warner and I. M. Willis. (2000) Repression of ribosome and tRNA synthesis in secretion- defective cells is signaled by a novel branch of the cell integrity pathway. *Mol. Cell. Biol.* **20**, 3843-3851.
- Liu, L., J. Liu, R. X. Qiu, X. G. Zhu, Z. Y. Dong and G. M. Tang. (2003) Improving heterologous gene expression in *Aspergillus niger* by introducing multiple copies of protein-binding sequence containing CCAAT to the promoter. *Lett. Appl. Microbiol.* **36**, 358-361.
- Liu, Y., P. Choudhury, C. M. Cabral and R. N. Sifers. (1999) Oligosaccharide modification in the early secretory pathway directs the selection of a misfolded glycoprotein for degradation by the proteasome. *J. Biol. Chem.* **274**, 5861-5867.
- Liu, Y., M. Schroder and R. J. Kaufman. (2000) Ligand-independent dimerization activates the stress response kinases IRE1 and PERK in the lumen of the endoplasmic reticulum. *J. Biol. Chem.* **275**, 24881-24885.
- Lodish, H. F. and N. Kong. (1990) Perturbation of cellular calcium blocks exit of secretory proteins from the rough endoplasmic reticulum. *J. Biol. Chem.* **265**, 10893-10899.
- Lowes, K. F., C. A. Shearman, J. Payne, D. MacKenzie, D. B. Archer, R. J. Merry and M. J. Gasson. (2000) Prevention of yeast spoilage in feed and food by the yeast mycocin HMK. *Appl. Environ. Microbiol.* **66**, 1066-1076.
- Ma, Y. J. and L. M. Hendershot. (2001) The unfolding tale of the unfolded protein response. *Cell* **107**, 827-830.
- MacCabe, A. P., J. vandenHomergh, J. Tilburn, H. N. Arst and J. Visser. (1996) Identification, cloning and analysis of the *Aspergillus niger* gene *pacC*, a wide domain regulatory gene responsive to ambient pH. *Mol. Gen. Genet.* **250**, 367-374.

- MacKenzie, D., D. J. Jeenes and D. B. Archer. (2004) Filamentous fungi as expression systems for heterologous proteins. *In: The Mycota II*, Ch.15 (U. Kuck, Ed.), Springer. 289- 315.
- MacKenzie, D. A., L. C. G. Gendron, D. J. Jeenes and D. B. Archer. (1994) Physiological optimization of secreted protein production *Aspergillus niger*. *Enz. Microb. Technol.* **16**, 276-280.
- Martin, T., R. V. Wöhner, S. Hummel, L. Willmitzer and W. Frommer. (1992) The GUS reporter system as a tool to study plant gene expression. *In Gus Protocols: Using the GUS gene as a reporter for gene expression*. Academic Press.
- Martinez, I. M. and M. J. Chrispeels. (2003) Genomic analysis of the unfolded protein response in *Arabidopsis* shows its connection to important cellular processes. *Plant Cell* **15**, 561-576.
- Marzioch, M., D. C. Henthorn, J. M. Herrmann, R. Wilson, D. Y. Thomas, J. J. M. Bergeron, R. C. E. Solari and A. Rowley. (1999) Erp1p and Erp2p, partners for Emp24p and Erv25p in a yeast p24 complex. *Mol. Biol. Cell* **10**, 1923-1938.
- Marzluff, W. F., E. C. Murphy and R. C. C. Huang. (1973) Transcription of ribonucleic-acid in isolated mouse myeloma nuclei. *Biochemistry* **12**, 3440-3446.
- Marzluff, W. F., E. C. Murphy and R. C. C. Huang. (1974) Transcription of genes for 5S ribosomal RNA and transfer RNA in isolated mouse myeloma cell nuclei. *Biochemistry* **13**, 3689-3696.
- Mattern, I. E., J. M. van noort, P. van den Berg, D. B. Archer, I. N. Roberts and C. A. M. J. J. van den Hondel. (1992) Isolation and characterization of mutants of *Aspergillus niger* deficient in extracellular proteases. *Mol. Gen. Genet.* **234**, 332-336.
- Mayer, M. P. and B. Bukau. (1998) Hsp70 chaperone systems: Diversity of cellular functions and mechanism of action. *Biol. Chem.* **379**, 261-268.
- McNabb, D. S., Y. Y. Xing and L. Guarente. (1995) Cloning of yeast Hap5, a novel subunit of a heterotrimeric complex required for CCAAT binding. *Genes Dev.* **9**, 47-58.
- Meyer, A. and E. Hartmann. (1997) The yeast SPC22/23 homolog Spc3p is essential for signal peptidase activity. *J. Biol. Chem.* **272**, 13159-13164.

- Mezzacasa, A. and A. Helenius. (2002) The transitional ER defines a boundary for quality control in the secretion of tsO45 VSV glycoprotein. *Traffic* **3**, 833-849.
- Michalak, M., J. M. R. Parker and M. Opas. (2002) Ca^{2+} signaling and calcium binding chaperones of the endoplasmic reticulum. *Cell Calcium* **32**, 269-278.
- Minetoki, T., C. Kumagai, K. Gomi, K. Kitamoto and K. Takahashi. (1998) Improvement of promoter activity by the introduction of multiple copies of the conserved region III sequence, involved in the efficient expression of *Aspergillus oryzae* amylose-encoding genes. *Appl. Microbiol. Biotechnol.* **50**, 459-467.
- Mizuno, K., T. Nakamura, T. Ohshima, S. Tanaka and H. Matsuo. (1989) Characterization of Kex2-encoded endopeptidase from yeast *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* **159**, 305-311.
- Molinari, M. and A. Helenius. (2000) Chaperone selection during glycoprotein translocation into the endoplasmic reticulum. *Science* **288**, 331-333.
- Moralejo, F. J., A. J. Watson, D. J. Jeenes, D. B. Archer and J. F. Martin. (2001) A defined level of protein disulfide isomerase expression is required for optimal secretion of thaumatin by *Aspergillus awamori*. *Mol. Gen. Genom.* **266**, 246-253.
- Morano, K. A., P. C. C. Liu and D. J. Thiele. (1998) Protein chaperones and the heat shock response in *Saccharomyces cerevisiae*. *Curr. Opin. Microbiol.* **1**, 197-203.
- Mori, K. (2000a) Tripartite management of unfolded proteins in the endoplasmic reticulum. *Cell* **101**, 451-454.
- Mori, K., T. Kawahara, H. Yoshida, H. Yanagi and T. Yura. (1996) Signalling from endoplasmic reticulum to nucleus: Transcription factor with a basic-leucine zipper motif is required for the unfolded protein-response pathway. *Genes Cells* **1**, 803-817.
- Mori, K., W. Z. Ma, M. J. Gething and J. Sambrook. (1993) A Transmembrane protein with a Cdc2+/Cdc28 related kinase activity is required for signaling from the ER to the nucleus. *Cell* **74**, 743-756.
- Mori, K., N. Ogawa, T. Kawahara, H. Yanagi and T. Yura. (1998) Palindrome with spacer of one nucleotide is characteristic of the cis-acting unfolded protein response element in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **273**, 9912-9920.

- Mori, K., N. Ogawa, T. Kawahara, H. Yanagi and T. Yura. (2000b) mRNA splicing-mediated C-terminal replacement of transcription factor Hac1p is required for efficient activation of the unfolded protein response. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 4660-4665.
- Mulder, H. J., M. Saloheimo, M. Penttilä and S. M. Madrid. (2004) The transcription factor HacA mediates the unfolded protein response in *Aspergillus niger*, and up-regulates its own transcription. *Mol. Genet. Genomics* **271**, 130-140.
- Munro, S. and H. R. B. Pelham. (1987) A C-terminal signal prevents secretion of luminal ER proteins. *Cell* **48**, 899-907.
- Nagata, K. (1998) Expression and function of heat shock protein 47: A collagen-specific molecular chaperone in the endoplasmic reticulum. *Matrix Biol.* **16**, 379-386.
- Ng, D. T., J. Brown and P. Walter. (1996) Signal sequences specify the targeting route to the endoplasmic reticulum membrane. *J. Cell Biol.* **134**, 269-278.
- Ngiam, C. (1998) Characterisation of a foldase in the protein secretory pathway of *Aspergillus niger*. Institute of Food Research. UK, The University of East Anglia: 209.
- Ngiam, C., D. J. Jeenes and D. B. Archer. (1997) Isolation and characterisation of a gene encoding protein disulphide isomerase, PdiA, from *Aspergillus niger*. *Curr. Genet.* **31**, 133-138.
- Ngiam, C., D. J. Jeenes, P. J. Punt, C. A. M. J. J. van den Hondel and D. B. Archer. (2000) Characterization of a foldase, protein disulfide isomerase A, in the protein secretory pathway of *Aspergillus niger*. *Appl. Environ. Microbiol.* **66**, 775-782.
- Nikawa, J. I. and S. Yamashita. (1992) Ire1 encodes a putative protein-kinase containing a membrane spanning domain and is required for inositol phototrophy in *Saccharomyces cerevisiae*. *Mol. Microbiol.* **6**, 1441-1446.
- Niwa, M. and P. Walter. (2000) Pausing to decide. *Proc. Natl. Acad. Sci. USA* **97**, 12396-12397.
- Noiva, R. (1999) Protein disulfide isomerase: The multifunctional redox chaperone of the endoplasmic reticulum. *Semin. Cell Dev. Biol.* **10**, 481-493.

Norgaard, P., V. Westphal, C. Tachibana, L. Alsoe, B. Holst and J. R. Winther. (2001) Functional differences in yeast protein disulfide isomerases. *J. Cell Biol.* **152**, 553-562.

Nyyssönen, E. and S. Keränen. (1995) Multiple roles of the cellulase Cbhl in enhancing production of fusion antibodies by the filamentous fungus *Trichoderma reesei*. *Curr. Genet.* **28**, 71-79.

Ortmann, B., J. Copeman, P. J. Lehner, B. Sadasivan, J. A. Herberg, A. G. Grandea, S. R. Riddell, R. Tampe, T. Spies, J. Trowsdale and P. Cresswell. (1997) A critical role for tapasin in the assembly and function of multimeric MHC class I-TAP complexes. *Science* **277**, 1306-1309.

Ou, W. J., P. H. Cameron, D. Y. Thomas and J. J. M. Bergeron. (1993) Association of folding intermediates of glycoproteins with calnexin during protein maturation. *Nature* **364**, 771-776.

Oxenboll, K. (1993) From Taxonomy and Genetics to Industrial Application. In Powell, K. A., Renwick, A., and Peberdy, J. F. (eds.), *The Genus Aspergillus*, Plenum Press, New York, 147-153,

Pakula, T. M., M. Laxell, A. Huuskonen, J. Uusitalo, M. Saloheimo and M. Penttilä. (2003) The effects of drugs inhibiting protein secretion in the filamentous fungus *Trichoderma reesei*. Evidence for down-regulation of genes that encode secreted proteins in the stressed cells. *J. Biol. Chem.* **278**, 45011-45020.

Pakula, T. M., M. Riikonen, J. Uusitalo, A. Huuskonen, M. Saloheimo, M. Penttilä, A. J. Watson, D. J. Jeenes and D. B. Archer. (2001) Improved method for production of secreted proteins by fungi. UK patent 20010272

Paloheimo, M., A. Mantyla, J. Kallio and P. Suominen. (2003) High-yield production of a bacterial xylanase in the filamentous fungus *Trichoderma reesei* requires a carrier polypeptide with an intact domain structure. *Appl. Environ. Microbiol.* **69**, 7073-7082.

Panzner, S., L. Dreier, E. Hartmann, S. Kostka and T. A. Rapoport. (1995) Posttranslational protein transport in yeast reconstituted with a purified complex of Sec proteins and Kar2p. *Cell* **81**, 561-570.

- Patil, C. and P. Walter. (2001) Intracellular signaling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. *Curr. Opin. Cell Biol.* **13**, 349-356.
- Pelham, H.R.B. (2000) Using sorting signals to retain proteins in the endoplasmic reticulum. *Meth. Enzymol.* **327**, 279-283.
- Petersen, K. L., J. Lehmbeck and T. Christensen. (1999) A new transcriptional activator for amylase genes in *Aspergillus*. *Mol. Gen. Genet.* **262**, 668-676.
- Pollard, M. G., K. J. Travers and J. S. Weissman. (1998) Ero1p: A novel and ubiquitous protein with an essential role in oxidative protein folding in the endoplasmic reticulum. *Mol. Cell.* **1**, 171-182.
- Powers, J. and C. Barlowe. (1998) Transport of Axl2p depends on Erv14p, an ER-vesicle protein related to the *Drosophila cornichon* gene product. *J. Cell Biol.* **142**, 1209-1222.
- Prieto, S., B. J. de la Cruz and I. E. Scheffler. (2000) Glucose-regulated turnover of mRNA and the influence of poly(A) tail length on half-life. *J. Biol. Chem.* **275**, 14155-14166.
- Ptashne, M. (1986) Gene regulation by proteins acting nearby and at a distance. *Nature* **322**, 697-701.
- Punt, P. J., A. Drint-Kuijvenhoven, B. C. Lokman, J. A. Spencer, D. Jeenes, D. A. Archer and C. A. M. J. J. van den Hondel. (2003) The role of the *Aspergillus niger* furin-type protease gene in processing of fungal proproteins and fusion proteins: Evidence for alternative processing of recombinant fusion-proteins. *J. Biotechnol.* **106**, 23-32.
- Punt, P. J., I. A. van Gemeren, J. Drint-Kuijvenhoven, J. G. M. Hessing, G. M. van Muijlwijk-Harteveld, A. Beijersbergen, C. T. Verrips and C. A. M. J. J. van den Hondel. (1998) Analysis of the role of the gene *bipA*, encoding the major endoplasmic reticulum chaperone protein in the secretion of homologous and heterologous proteins in black *Aspergilli*. *Appl. Microbiol. Biotechnol.* **50**, 447-454.
- Qiu, R. X., X. G. Zhu, L. Liu and G. M. Tang. (2002) Detection of a protein, AngCP, which binds specifically to the three upstream regions of *glaA* gene in *A. niger* T21. *Sci. China Ser. C-Life Sci.* **45**, 527-537.

- Rao, R. V., E. Hermel, S. Castro-Obregon, G. del Rio, L. M. Ellerby, H. M. Ellerby and D. E. Bredesen. (2001) Coupling endoplasmic reticulum stress to the cell death program. Mechanism of caspase activation. *J. Biol. Chem.* **276**, 33869-33874.
- Rapoport, T. A., B. Junnickel and U. Kutay. (1996) Protein transport across the eukaryotic endoplasmic reticulum and bacterial inner membranes. *Annu. Rev. Biochem.* **65**, 271-303.
- Roberg, K. J., M. Crotwell, P. Espenshade, R. Gimeno and C. A. Kaiser. (1999) LST1 is a SEC24 homologue used for selective export of the plasma membrane ATPase from the endoplasmic reticulum. *J. Cell Biol.* **145**, 659-672.
- Roberts, I. N., D. J. Jeenes, D. A. MacKenzie, A. P. Wilkinson, I. G. Sumner and D. B. Archer. (1992) Heterologous gene expression in *Aspergillus niger* : A glucoamylase-porcine pancreatic prophospholipase-a(2) fusion protein is secreted and processed to yield mature enzyme. *Gene* **122**, 155-161.
- Robinson, A. S., V. Hines and K. D. Wittrup. (1994) Protein disulfide isomerase overexpression increases secretion of foreign proteins in *Saccharomyces cerevisiae*. *Biotechnology* **12**, 381-384.
- Romanos, M. A., A. J. Makoff, N. F. Fairweather, K. M. Beesley, D. E. Slater, F. B. Rayment, M. M. Payne and J. J. Clare. (1991) Expression of tetanus toxin fragment-C in yeast: Gene synthesis is required to eliminate fortuitous polyadenylation sites in AT-rich DNA. *Nucleic Acids Res.* **19**, 1461-1467.
- Rose, M. D., L. M. Misra and J. P. Vogel. (1989) *Kar2*, a karyogamy gene, is the yeast homolog of the mammalian *Bip/Grp78* gene. *Cell* **57**, 1211-1221.
- Rosenberg, C., E. Wong, E. Petty, A. Bale, Y. Tsujimoto, N. Harris and A. Arnold. (1991) PRAD1, a candidate BLC1 oncogene: Mapping and expression in centrocytic lymphoma. *Proc. Natl. Acad. Sci. USA* **88**, 9638-9642.
- Rüegsegger, U., J. H. Leber and P. Walter. (2001) Block of HAC1 mRNA translation by long-range base pairing is released by cytoplasmic splicing upon induction of the unfolded protein response. *Cell* **107**, 103-114.
- Ruiz-Duenas, F. J., T. A. L. Chau, M. Perez-Boada, M. J. Martinez and A. T. Martinez. (2001) Heterologous expression of a ligninolytic versatile peroxidase from *Pleurotus eryngii* in *Aspergillus* species. *J. Inorg. Biochem.* **86**, 411-411.

- Rutishauser, J. and M. Spiess. (2002) Endoplasmic reticulum storage diseases. *Swiss Med. Wkly.* **132**, 211-222.
- Sachs, M. S. (1998) Posttranscriptional control of gene expression in filamentous fungi. *Fungal Genet. Biol.* **23**, 117-125.
- Sakaguchi, M. (1997) Eukaryotic protein secretion. *Curr. Opin. Biotechnol.* **8**, 595-601.
- Sakaguchi, M., R. Tomiyoshi, T. Kuroiwa, K. Mihara and T. Omura. (1992) Functions of signal and signal-anchor sequences are determined by the balance between the hydrophobic segment and the N-terminal charge. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 16-19.
- Saloheimo, M., M. Lund and M. E. Penttilä. (1999) The protein disulphide isomerase gene of the fungus *Trichoderma reesei* is induced by endoplasmic reticulum stress and regulated by the carbon source. *Mol. Gen. Genet.* **262**, 35-45.
- Saloheimo, M., M. Valkonen and M. Penttilä. (2003) Activation mechanisms of the Hac1-mediated unfolded protein response in filamentous fungi. *Mol. Microbiol.* **47**, 1149-1161.
- Saloheimo, M., H. M. Wang, M. Valkonen, T. Vasara, A. Huuskonen, M. Riikonen, T. Pakula, M. Ward and M. Penttilä. (2004) Characterization of secretory genes *ypt1/yptA* and *nsf1/insfA* from two filamentous fungi: Induction of secretory pathway genes of *Trichoderma reesei* under secretion stress conditions. *Appl. Environ. Microbiol.* **70**, 459-467.
- Sambrook, J., E. F. Fritsch and T. Maniatis. (1989) Molecular Cloning: A Laboratory Manual. New York, Cold Spring Harbor Laboratory Press.
- Santi, C., S. Svistoonoff, L. Constans, F. Auguy, E. Duhoux, D. Bogusz and C. Franche. (2003) Choosing a reporter for gene expression studies in transgenic actinorhizal plants of the Casuarinaceae family. *Plant Soil* **254**, 229-237.
- Schlaman, H. R. M., E. Risseuw, M. E. I. Frankevandijk and P. J. J. Hooykaas. (1994) Nucleotide sequence corrections of the *uidA* open reading frame encoding β -glucuronidase. *Gene* **138**, 259-260.

- Schröder, M., J. S. Chang and R. J. Kaufman. (2000) The unfolded protein response represses nitrogen-starvation induced developmental differentiation in yeast. *Genes Dev.* **14**, 2962-2975.
- Schröder, M., R. Clark and R. J. Kaufman. (2003) *IRE1*- and *HAC1*-independent transcriptional regulation in the unfolded protein response of yeast. *Mol. Microbiol.* **49**, 591-606.
- Schultz, L. D., H. Z. Markus, K. J. Hofmann, D. L. Montgomery, C. T. Dunwiddie, P. J. Kniskern, R. B. Freedman, R. W. Ellis, and M. F. Tuite. (1994) Using molecular genetics to improve the production of recombinant proteins by the yeast *Saccharomyces cerevisiae*. *Ann. N. Y. Acad. Sci.* **721**:148-157.
- Schuren, F. H. J., T. R. van der Lende and J. G. H. Wessels. (1993) Fruiting genes of *Schizophyllum commune* are transcriptionally regulated. *Mycol. Res.* **97**, 538-542.
- Schuster, E., N. Dunn-Coleman, J. C. Frisvad and P. W. M. van Dijck. (2002) On the safety of *Aspergillus niger*. *Appl. Microbiol. Biotechnol.* **59**, 426-435.
- Scorer, C. A., R. G. Buckholz, J. J. Clare and M. A. Romanos. (1993) The intracellular production and secretion of HIV-1 envelope protein in the methylotrophic yeast *Pichia pastoris*. *Gene* **136**, 111-119.
- Semenza, J. C., K. G. Hardwick, N. Dean and H. R. B. Pelham. (1990) *ERD2*, a yeast gene required for the receptor-mediated retrieval of luminal ER proteins from the secretory pathway. *Cell* **61**, 1349-1357.
- Shamu, C., J. S. Cox and P. Walter. (1994) The unfolded protein response pathway in yeast. *Trends in Cell Biol* **4**, 56-60.
- Shamu, C. and P. Walter. (1996) Oligomerization and phosphorylation of the Ire1p kinase during intracellular signaling from the endoplasmic reticulum to the nucleus. *EMBO J.* **15**, 3028-3039.
- Shen, X. H., K. Z. Zhang and R. J. Kaufman. (2004) The unfolded protein response: A stress signaling pathway of the endoplasmic reticulum. *J. Chem. Neuroanat.* **28**, 79-92.
- Shorrosh, B. S. and R. A. Dixon. (1992) Molecular characterization and expression of an Alfalfa protein with sequence similarity to mammalian Erp72, a glucose

regulated endoplasmic reticulum protein containing active site sequences of protein disulfide isomerase. *Plant J.* **2**, 51-58.

Sidrauski, C. and P. Walter. (1997) The transmembrane kinase Ire1p is a site-specific endonuclease that initiates mRNA splicing in the unfolded protein response. *Cell* **90**, 1031-1039.

Silberstein, S., G. Schlenstedt, P. A. Silver and R. Gilmore. (1998) A role for the DnaJ homologue Scj1p in protein folding in the yeast endoplasmic reticulum. *J. Cell Biol.* **143**, 921-933.

Skjot, M., S. Kauppinen, L. V. Kofod, C. Fuglsang, M. Pauly, H. Dalboge and L. N. Andersen. (2001) Functional cloning of an endo-arabinanase from *Aspergillus aculeatus* and its heterologous expression in *Aspergillus oryzae* and tobacco. *Mol. Genet. Genomics* **265**, 913-921.

Srivastava, S. P., N. Q. Chen, Y. X. Liu and J. L. Holtzman. (1991) Purification and characterization of a new isozyme of thiol protein-disulfide oxidoreductase from rat hepatic microsomes. Relationship of this isozyme to cytosolic phosphatidylinositol-specific phospholipase-C form 1A. *J. Biol. Chem.* **266**, 20337-20344.

Stirling, C. J. (1999) Protein targeting to the endoplasmic reticulum in yeast. *Microbiology-(UK)* **145**, 991-998.

Struhl, K. (1989) Molecular mechanisms of transcriptional regulation in yeast. *Annu. Rev. Biochem.* **58**, 1051-1077.

Suto, M. and F. Tomita. (2001) Induction and catabolite repression mechanisms of cellulase in fungi. *J. Biosci. Bioeng.* **92**, 305-311.

Svetina, M., N. Krasevec, V. Gaberc-Porekar and R. Komel. (2000) Expression of catalytic subunit of bovine enterokinase in the filamentous fungus *Aspergillus niger*. *J. Biotechnol.* **76**, 245-251.

Tachibana, C. and T. H. Stevens. (1992) The yeast *EUG1* gene encodes an endoplasmic reticulum protein that is functionally related to protein disulfide isomerase. *Mol. Cell. Biol.* **12**, 4601-4611.

Tani, S., T. Itoh, M. Kato, T. Kobayashi and N. Tsukagoshi. (2001b) *In vivo* and *in vitro* analyses of the AmyR binding site of the *Aspergillus nidulans* *agdA* promoter;

Requirement of the CGG direct repeat for induction and high affinity binding of AmyR. *Biosci. Biotechnol. Biochem.* **65**, 1568-1574.

Tani, S., Y. Katsuyama, T. Hayashi, H. Suzuki, M. Kato, K. Gomi, T. Kobayashi and N. Tsukagoshi. (2001a) Characterization of the *amyR* gene encoding a transcriptional activator for the amylase genes in *Aspergillus nidulans*. *Curr. Genet.* **39**, 10-15.

Teasdale, R. D. and M. R. Jackson. (1996) Signal-mediated sorting of membrane proteins between the endoplasmic reticulum and the Golgi apparatus. *Annu. Rev. Cell Dev. Biol.* **12**, 27-54.

Thompson, S. A., E. J. Golightly and D. S. Yaver. (1995) Nucleotide sequence of the *Aspergillus niger* *srpA* gene. *Gene* **167**, 337-338.

Toda, T., M. Sano, M. Honda, O. Rimoldi, Y. Yang, M. Yamamoto, K. Takase, K. Hirozumi, K. Kitamoto, T. Minetoki, K. Gomi and M. Machida. (2001) Deletion analysis of the enolase gene (*enoA*) promoter from the filamentous fungus *Aspergillus oryzae*. *Curr. Genet.* **40**, 260-267.

Torigoi, E., I. M. Bennani-Baiti, C. Rosen, K. Gonzalez, P. Morcillo, M. Ptashne and D. Dorsett. (2000) Chip interacts with diverse homeodomain proteins and potentiates Bicoid activity *in vivo*. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 2686-2691.

Travers, K. J., C. K. Patil, L. Wodicka, D. J. Lockhart, J. S. Weissman and P. Walter. (2000) Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER- associated degradation. *Cell* **101**, 249-258.

Trilla, J. A., A. Duran and C. Roncero. (1999) Chs7p, a new protein involved in the control of protein export from the endoplasmic reticulum that is specifically engaged in the regulation of chitin synthesis in *Saccharomyces cerevisiae*. *J. Cell Biol.* **145**, 1153-1163.

Tsuchiya, K., K. Gomi, K. Kitamoto, C. Kumagai and G. Tamura. (1993) Secretion of calf chymosin from the filamentous fungus *Aspergillus oryzae*. *Appl. Microbiol. Biotechnol.* **40**, 327-332.

Tsuchiya, K., T. Nagashima, Y. Yamamoto, K. Gomi, K. Kitamoto, C. Kumagai and G. Tamura. (1994) High level secretion of calf chymosin using a glucoamylase-

prochymosin fusion gene in *Aspergillus oryzae*. *Biosci. Biotechnol. Biochem.* **58**, 895-899.

Valkonen, M., M. Penttilä and M. Saloheimo. (2003a) Effects of inactivation and constitutive expression of the unfolded protein response pathway on protein production in the yeast *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **69**, 2065-2072.

Valkonen, M., M. Ward, H. M. Wang, M. Penttilä and M. Saloheimo. (2003b) Improvement of foreign-protein production in *Aspergillus niger* var. *awamori* by constitutive induction of the unfolded protein response. *Appl. Environ. Microbiol.* **69**, 6979-6986.

van den Brink, J. M., P. J. Punt, R. F. M. van Gorcom and C. A. M. J. J. van den Hondel. (2000) Regulation of expression of the *Aspergillus niger* benzoate para-hydroxylase cytochrome P450 system. *Mol. Gen. Genet.* **263**, 601-609.

van den Hombergh, J., G. Jarai, F. P. Buxton and J. Visser. (1994) Cloning, characterization and expression of *pepF*, a gene encoding a serine carboxypeptidase from *Aspergillus niger*. *Gene* **151**, 73-79.

van den Hombergh, J., P. J. I. van den Vondervoort, L. FraissinetTachet and J. Visser. (1997) *Aspergillus* as a host for heterologous protein production: The problem of proteases. *Trends Biotechnol.* **15**, 256-263.

van Dijck, P. W. M., G. C. M. Selten and R. A. Hempenius. (2003) On the safety of a new generation of DSM *Aspergillus niger* enzyme production strains. *Regul. Toxicol. Pharmacol.* **38**, 27-35.

van Gemeren, I. A., P. J. Punt, A. Drint Kuyvenhoven, M. P. Broekhuijsen, A. van Hoog, A. Beijersbergen, C. T. Verrips and C. A. M. J. J. van den Hondel. (1997) The ER chaperone encoding *bipA* gene of black *Aspergilli* is induced by heat shock and unfolded proteins. *Gene* **198**, 43-52.

van Gorcom, R.F.M., Punt, P.J. and van den Hondel, C.A.M.J.J. (1994) Heterologous gene expression in *Aspergillus*. In K. A. Powell, A. Renwick and J.F. Peberdy (eds.), *The Genus Aspergillus, from Taxonomy and Genetics to Industrial Application*, FEMS Symposium 69, , Plenum Press, New York, pp 241-250.

- van Hartingsveldt, W., I. E. Mattern, C. M. J. van zeijl, P. H. Pouwels and C. A. M. J. J. van den Hondel. (1987) Development of a homologous transformation system for *Aspergillus niger* based on the *pyrG* gene. *Mol. Gen. Genet.* **206**, 71-75.
- Varga, J., F. Kevei, A. Vriesema, F. Debets, Z. Kozakiewicz and J. H. Croft. (1994) Mitochondrial DNA Restriction Fragment Length Polymorphisms in field isolates of the *Aspergillus niger* aggregate. *Can. J. Microbiol.* **40**, 612-621.
- Verdoes, J. C., P. J. Punt, J. M. Schrickx, H. W. Vanverseveld, A. H. Stouthamer and C. A. M. J. J. van den Hondel. (1993) Glucoamylase overexpression in *Aspergillus niger* molecular genetic analysis of strains containing multiple copies of the *glaA* gene. *Transgenic Research* **2**, 84-92.
- Verdoes, J. C., P. J. Punt, A. H. Stouthamer and C. A. M. J. J. van den Hondel. (1994) The effect of multiple copies of the upstream region on expression of the *Aspergillus niger* glucoamylase-encoding gene. *Gene* **145**, 179-187.
- Vilela, C. and J. E. G. McCarthy. (2003) Regulation of fungal gene expression via short open reading frames in the mRNA 5' untranslated region. *Mol. Microbiol.* **49**, 859-867.
- Voges, D., P. Zwickl and W. Baumeister. (1999) The 26S proteasome: A molecular machine designed for controlled proteolysis. *Annu. Rev. Biochem.* **68**, 1015-1068.
- Voigt, S., B. Jungnickel, E. Hartmann and T. A. Rapoport. (1996) Signal sequence-dependent function of the TRAM protein during early phases of protein transport across the endoplasmic reticulum membrane. *J. Cell Biol.* **134**, 25-35.
- von Heijne, G. (1990) The signal peptide. *J. Membr. Biol.* **115**, 195-201.
- Walter, P. and A. E. Johnson. (1994) Signal sequence recognition and protein targeting to the endoplasmic reticulum membrane. *Annu. Rev. Cell Biol.* **10**, 87-119.
- Wang, H., J. Entwistle, E. Morlon, D. B. Archer, J. F. Peberdy, M. Ward and D. J. Jeenes. (2003) Isolation and characterisation of a calnexin homologue, *clxA*, from *Aspergillus niger*. *Mol. Genet. Genomics* **268**, 684-691.
- Wang, H. and M. Ward. (2000) Molecular characterization of a PDI-related gene *prpA* in *Aspergillus niger* var. *awamori*. *Curr. Genet.* **37**, 57-64.

- Wang, X. Z., H. P. Harding, Y. H. Zhang, E. M. Jolicoeur, M. Kuroda and D. Ron. (1998) Cloning of mammalian Ire1 reveals diversity in the ER stress responses. *EMBO J.* **17**, 5708-5717.
- Ward, M., L. J. Wilson and K. H. Kodama. (1993) Use of *Aspergillus* overproducing mutants, cured for integrated plasmid, to overproduce heterologous proteins. *Appl. Microbiol. Biotechnol.* **39**, 738-743.
- Ward, M., L. J. Wilson, K. H. Kodama, M. W. Rey and R. M. Berka. (1990) Improved production of chymosin in *Aspergillus* by expression as a glucoamylase-chymosin fusion. *Biotechnology* **8**, 435-440.
- Ward, P., C. S. Piddington, G. A. Cunningham, X. D. Zhou, R. D. Wyatt and O. M. Conneely. (1995) A System for production of commercial quantities of human lactoferrin. A broad-spectrum natural antibiotic. *Biotechnology* **13**, 498-503.
- Ware, F. E., A. Vassilakos, P. A. Peterson, M. R. Jackson, M. A. Lehrman and D. B. Williams. (1995) The molecular chaperone calnexin binds Glc(1)Man(9)Glcna(2) oligosaccharide as an initial step in recognizing unfolded glycoproteins. *J. Biol. Chem.* **270**, 4697-4704.
- Watanabe, A., I. Fujii, H. F. Tsai, Y. C. Chang, K. J. Kwon-Chung and Y. Ebizuka. (2000) *Aspergillus fumigatus alb1* encodes naphthopyrone synthase when expressed in *Aspergillus oryzae*. *FEMS Microbiol. Lett.* **192**, 39-44.
- Watson, A. J., J. Worley, R. M. Elliott, D. J. Jeenes and D. B. Archer. (2000) Cloning stress-induced genes from *Aspergillus niger* using polymerase chain reaction augmented subtractive hybridization. *Anal. Biochem.* **277**, 162-165.
- Welihinda, A., W. Tirasophon, S. R. Green and R. J. Kaufman. (1998) Protein serine/threonine phosphatase Ptc2p negatively regulates the unfolded protein response by dephosphorylating Ire1p kinase. *Mol. Cell. Biol.* **18**, 1967-1977.
- Welihinda, A., W. Tirasophon and R. J. Kaufman. (2000) The transcriptional co-activator ADA5 is required for HAC1 mRNA processing *in vivo*. *J. Biol. Chem.* **275**, 3377-3381.
- Wiebe, M. G., A. Karandikar, G. D. Robson, A. P. J. Trinci, J. L. F. Candia, S. Trappe, G. Wallis, U. Rinas, P. M. F. Derkx, S. M. Madrid, H. Sisniega, I. Faus, R. Montijn, C. A. M. J. J. van den Hondel and P. J. Punt. (2001) Production of tissue plasminogen activator (t-PA) in *Aspergillus niger*. *Biotechnol. Bioeng.* **76**, 164-174.

- Wilcox, C. A., K. Redding, R. Wright and R. S. Fuller. (1992) Mutation of a tyrosine localization signal in the cytosolic tail of yeast Kex2p protease disrupts Golgi retention and results in default transport to the vacuole. *Mol. Biol. Cell* **3**, 1353-1371.
- Wileman, T., L. P. Kane, G. R. Carson and C. Terhorst. (1991) Depletion of cellular calcium accelerates protein-degradation in the endoplasmic reticulum. *J. Biol. Chem.* **266**, 4500-4507.
- Woycechowsky, K. J. and R. T. Raines. (2000) Native disulfide bond formation in proteins. *Curr. Opin. Chem. Biol.* **4**, 533-539.
- YaDeau, J., C. Klein and G. Blobel. (1991) Yeast signal peptidase contains a glycoprotein and the Sec11 gene product. *Proc. Natl. Acad. Sci. USA* **88**, 517-521.
- Ye, J., R. B. Rawson, R. Komuro, X. Chen, U. P. Dave, R. Prywes, M. S. Brown and J. L. Goldstein. (2000) ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. *Mol. Cell.* **6**, 1355-1364.
- Yoshida, H., K. Haze, H. Yanagi, T. Yura and K. Mori. (1998) Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. Involvement of basic leucine zipper transcription factors. *J. Biol. Chem.* **273**, 33741-33749.
- Yoshida, H., T. Matsui, A. Yamamoto, T. Okada and K. Mori. (2001a) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* **107**, 881-891.
- Yoshida, H., T. Okada, K. Haze, H. Yanagi, T. Yura, M. Negishi and K. Mori. (2001b) Endoplasmic reticulum stress-induced formation of transcription factor complex ERSF including NF-Y (CBF) and activating transcription factors 6 α and 6 β that activates the mammalian unfolded protein response. *Mol. Cell. Biol.* **21**, 1239-1248.
- Zapun, A., N. J. Darby, D. C. Tessier, M. Michalak, J. J. M. Bergeron and D. Y. Thomas. (1998) Enhanced catalysis of ribonuclease B folding by the interaction of calnexin or calreticulin with ERp57. *J. Biol. Chem.* **273**, 6009-6012.
- Zapun, A., C. A. Jakob, D. Y. Thomas and J. J. M. Bergeron. (1999) Protein folding in a specialized compartment: The endoplasmic reticulum. *Struct. Fold. Des.* **7**, R173-R182.

Zar, J. H. (1996) Biostatistical Analysis. New Jersey, USA, Prentice Hall.

Zerangue, N., B. Schwappach, Y. N. Jan and L. Y. Jan. (1999) A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane K-ATP channels. *Neuron* **22**, 537-548.

Zhu, X. G., R. X. Qiu, L. Liu and G. M. Tang. (2004) Binding of trans-acting protein AngCP to the CCAAT-containing motifs in *Aspergillus niger glaA* promoter. *Prog. Nat. Sci.* **14**, 338-343.

Endoplasmic reticulum stress leads to the selective transcriptional downregulation of the glucoamylase gene in *Aspergillus niger*

Hashem Al-Sheikh,¹ Adrian J. Watson,¹ Georgina A. Lacey,² Peter J. Punt,³ Donald A. MacKenzie,² David J. Jeenes,² Tiina Pakula,⁴ Merja Penttilä,⁴ Marcos J. C. Alcocer¹ and David B. Archer^{1*}

¹School of Biology, University of Nottingham, University Park, Nottingham NG7 2RD, UK.

²Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, UK.

³TNO Nutrition and Food Research Institute, PO Box 360, 3700 AJ Zeist, the Netherlands.

⁴VTT, Technical Research Centre of Finland, VTT Biotechnology and Food Research, PO Box 1500, Espoo FIN-02044 VTT, Finland.

Summary

We describe a new endoplasmic reticulum (ER)-associated stress response in the filamentous fungus *Aspergillus niger*. The inhibition of protein folding within the ER leads to cellular responses known collectively as the unfolded protein response (UPR) and we show that the selective transcriptional downregulation of the gene encoding glucoamylase, a major secreted protein, but not two non-secreted proteins, is an additional consequence of ER stress. The transcriptional downregulation effect is shown by nuclear run-on studies to be at the level of transcription, rather than mRNA stability, and is found to be mediated through the promoter of *glaA* in a region more than 1 kb upstream of the translational start. The inhibition of protein folding in the ER can be induced in a variety of ways. We examined the effects of dithiothreitol (DTT), a reducing agent that causes the formation of unfolded proteins. Although a general downregulation of transcription was seen with DTT treatment, we show that selective downregulation was observed with the *glaA* gene compared with genes encoding the non-secreted proteins γ -actin and glyceraldehyde 3'-phosphate dehydrogenase. The DTT-treated fungal cells also showed evidence for the induction of the UPR because expression of *bipA* and

pdiA, encoding an ER-resident chaperone and foldase, respectively, are upregulated and splicing of *hacA*, the gene encoding the transcription factor responsible for induction of the UPR, occurs allowing the production of an active HacA protein. As a preliminary attempt to investigate if the transcriptional downregulation effect was mediated through HacA (i.e. part of the UPR), we examined ER stress induced through antisense technology to lower the level of PDI in the ER of *A. niger*. Although the transcription of *glaA* was attenuated in that strain of *A. niger*, UPR was not evident, suggesting that the transcriptional downregulation mechanism is controlled differently from the UPR.

Introduction

The secretion of proteins is a key facet of the lifestyles of microorganisms and higher eukaryotes. Saprophytic filamentous fungi such as *Aspergillus niger* and *Trichoderma reesei* depend on the efficient secretion of enzymes to degrade polymeric organic matter and to provide the substrates for energy production and cellular growth. Filamentous fungi are also widely used in the biotechnology industries to produce enzymes for a variety of uses (Archer and Peberdy, 1997). Inefficient transit of proteins, whether homologous or heterologous, destined for secretion through the endoplasmic reticulum (ER), could be a significant bottleneck in production of commercially viable levels of protein (Conesa *et al.*, 2001).

In recent years, feedback mechanisms have been described in eukaryotic systems that allow the cell to sense the state of the lumen of the ER and respond to changes in the normal functioning of this specialized compartment for protein folding and processing. The first of these mechanisms is the unfolded protein response (UPR) which increases the transcriptional activity of genes encoding chaperones and folding catalysts in response to the accumulation of unfolded proteins in the ER (Shamu *et al.*, 1994). The response of yeast and mammalian cells to increased levels of unfolded proteins in the ER has been reviewed by Mori (2000) and the specific role of the UPR by Patil and Walter (2001). ER stress in mammalian cells leads to phosphorylation of the eukaryotic initiation factor 2 α which downregulates translation

Accepted 25 May, 2004. *For correspondence. E-mail: david.archer@nottingham.ac.uk; Tel. (+44) 115 9513313; Fax (+44) 115 9513251.

(Harding *et al.*, 1999) but this effect is not reported in yeasts or filamentous fungi. The sensors for both of these mechanisms are *trans*-membrane proteins that reside in the ER membrane and which dimerize when unfolded proteins are present in the ER. In addition, a third response emanating from the ER exists that is termed ER-associated (protein) degradation (ERAD) which has been reviewed by Bonifacino and Weissman (1998). This pathway targets terminally misfolded proteins for degradation in the proteasome by retrotranslocation from the ER to the cytosol and ubiquitination to target the protein to the proteasome. Although the sensor involved in ERAD is not known, research suggests that the UPR and ERAD are very closely integrated (Travers *et al.*, 2000).

In the current study we have used dithiothreitol (DTT), which is known to induce the UPR, and antisense *pdiA* to reduce the level of the foldase, protein disulphide isomerase (PDI), to probe the effects of unfolded proteins on the filamentous fungus *A. niger*. Previous studies have shown that the UPR is active in *A. niger* (van Gemeren *et al.*, 1997; Ngiam *et al.*, 1997; 2000) and is induced by DTT, tunicamycin (an inhibitor of N-glycosylation of secreted proteins) and by the secretion of heterologous proteins. Similar effects have also been shown in *T. reesei* (Saloheimo *et al.*, 1999). The UPR in these fungi is characterized by the transcriptional upregulation of genes encoding the principal chaperone BiP and foldases such as PDI. The transcription factor that mediates the UPR, termed HacA/Hac1, is encoded by an mRNA that is processed in an analogous but not identical manner (Saloheimo *et al.*, 2003) to that shown in *Saccharomyces cerevisiae* (Kawahara *et al.*, 1998; Patil and Walter, 2001). In *A. niger*, the mRNA that is translated to the HacA product has a shortened 5'-untranslated region (230 bp) and is spliced within the open reading frame (ORF) by removal of a short (20 bp) sequence (Mulder *et al.*, 2004), which we term below as an 'intron'. In addition to the UPR, we show here a previously undescribed response to ER stress in *A. niger* that acts to specifically downregulate the transcription of the glucoamylase gene (*glaA*). The transcriptional downregulation of genes encoding secreted proteins under ER stress has previously been shown in *T. reesei* (Pakula *et al.*, 2003) and *Arabidopsis thaliana* (Martinez and Chrispeels, 2003). We show here that the observed effect is because of transcription, and not mRNA degradation, and we define a mediating region of the *glaA* promoter.

Results

ER stress induced by DTT

The effect of DTT on steady-state mRNA levels was assessed for glucoamylase (*glaA*) and the ER-resident

chaperone BiP (*bipA*) (Fig. 1) as well as the gene encoding the secreted protease aspergillopepsin (*pepA*) and the foldase PDI (*pdiA*) (data not shown). Figure 1(A) shows the effect on the steady state mRNA levels for the *glaA* gene over this period. Note that the steady accumulation of the *glaA* transcript relative to actin, in the absence of DTT, probably indicates a relative stability of the *glaA* mRNA. The data show that, in the DTT-treated cultures, the level of *glaA* mRNA declines steadily with time, with a half-life of approximately 80 min. This correlates well with other data from medium exchange experiments which suggest that the half-life of *A. niger glaA* mRNA in the absence of *de novo* synthesis of *glaA* mRNA is about the same (data not shown). These data suggest that DTT treatment inhibits the transcription of *glaA* and a similar response was shown for *pepA*. Figure 1(B) confirms that DTT treatment rapidly raised the steady-state level of the *bipA* mRNA (Ngiam *et al.*, 1997; 2000) showing that DTT induced the UPR. The mRNA level of *pdiA* was induced in a similar manner (data not shown). Both *bipA* and *pdiA* showed a rapid transcriptional response to the addition of the stress agent. This response is not transient because the levels remain high during the course of the study period. It is not known whether this longevity is because of the production of mRNA for an extended period caused by persistence of the stress agent or because of long half-lives of the transcripts involved. Figure 1(C) shows dry weight data for the DTT-treated and untreated samples. There does not appear to be a significant difference between the DTT-treated samples and the water controls. Biomass declined slightly over this period and may represent unavoidable effects of the experimental procedure such as the need to have the cultures stationary while adding the stress agent or water as control (the viability of neither culture was impaired over longer periods).

ER stress in an antisense *pdiA* strain of *A. niger*

Figure 2 shows data obtained from a comparison of *A. niger* AS1.1 (Ngiam *et al.*, 2000), which contains between four and six copies of a *pdiA* antisense sequence under the control of the strongly inducible glucoamylase promoter, to the parental strain AB4.1 (van Hartingsveldt *et al.*, 1987) when grown on starch as an inducing carbon source. We have previously demonstrated that levels of PDI protein in microsomal preparations are significantly lower in the inducible antisense strain than in the parental strain, and that secreted glucoamylase levels are also considerably lower (Ngiam *et al.*, 2000). Figure 2(A) shows the effect of antisense *pdiA* on the mRNA levels of the *glaA* gene over a period of batch growth. It can be seen that from the first time point at 24 h, the levels of *glaA* mRNA in the inducible antisense *pdiA* strain show a gradual decline while those for the parent increase relative

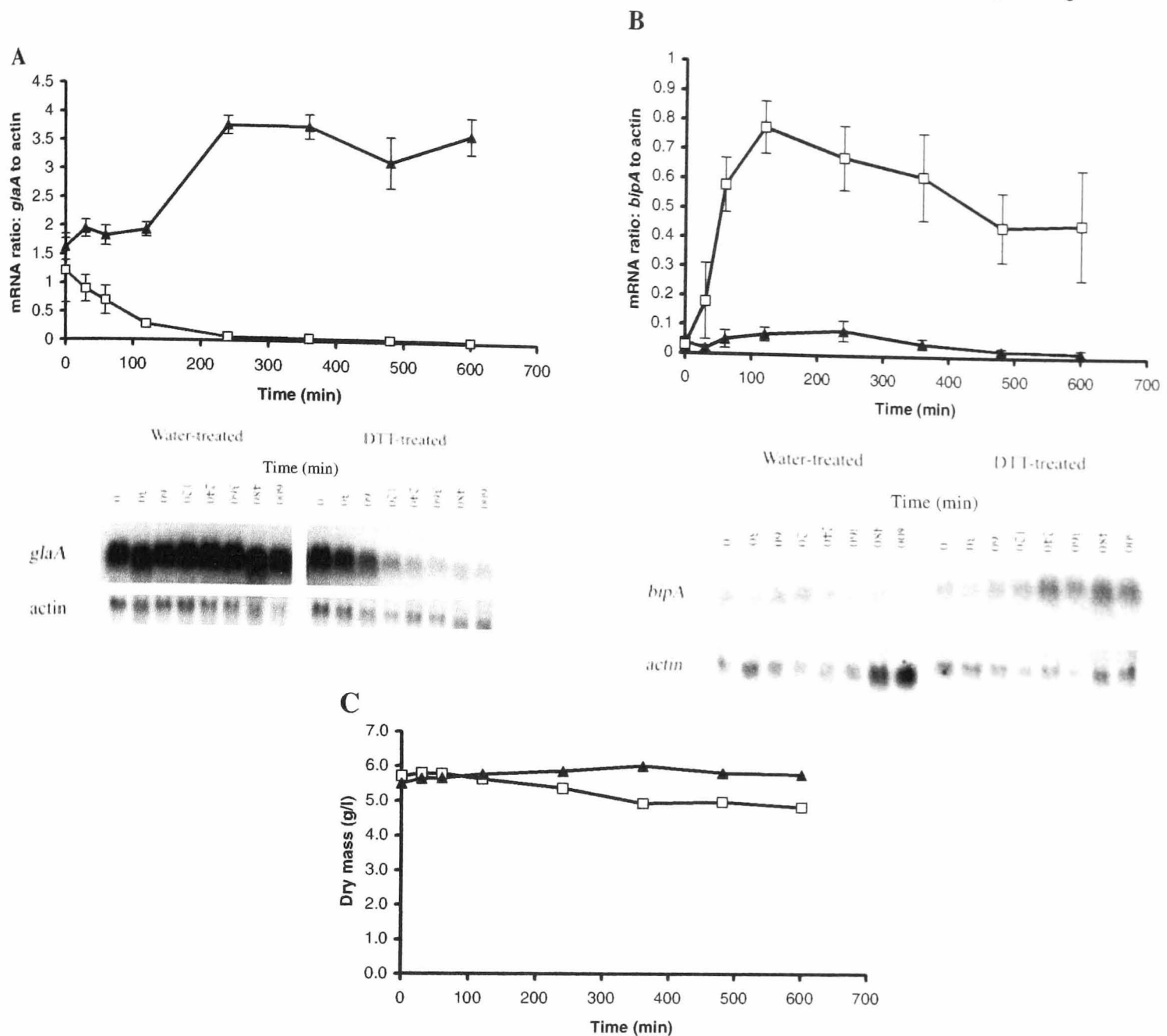


Fig. 1. The effects of DTT on the transcription of various genes from *A. niger*. Steady-state transcript levels of *glaA* (A) and *bipA* (B) and actin were measured by Northern blotting and quantified by phosphorimaging. In each of the figures there are two traces, the DTT-treated cultures (□) and the water-treated controls (▲). Below the graphs are representative Northern blots. The time on the lower axis represents the time in minutes after addition of the stress agent. The values for the amounts of mRNA are normalized values to the levels of the γ -actin transcript and are not directly comparable to each other. The data represent averages from three replicates from each time-point. C shows the dry weight of mycelium over the period of study for stress response.

to γ -actin. The levels of *glaA* mRNA in the parental strain increase up to 60 h relative to the levels of γ -actin which is used to normalize the loadings on the Northern blots. This may be due in part (as in Fig. 1A) to the long half-life of the *glaA* mRNA which would mean that the rate of breakdown of the mRNA is significantly slower than the rate of production, giving rise to an increasing population of this species of mRNA over time. The level of transcripts of the *pepA* gene encoding aspergillopepsin were similarly decreased in the inducible antisense *pdiA* strain (data not shown). The kinetics of growth and final yield of

biomass were not significantly different in the parental strain and the inducible antisense *pdiA* strain in this medium (Fig. 2B). The DTT-treatment was a time-limited stress experiment whereas the inducible antisense *pdiA* study was a longer growth-related study, hence the kinetic effects are different.

In the initial experiments expression of both the antisense *pdiA* gene and the native *glaA* gene were driven by the *glaA* promoter, raising the possibility that transcriptional effects might arise because of competition for the same transcription factors. We therefore exchanged the

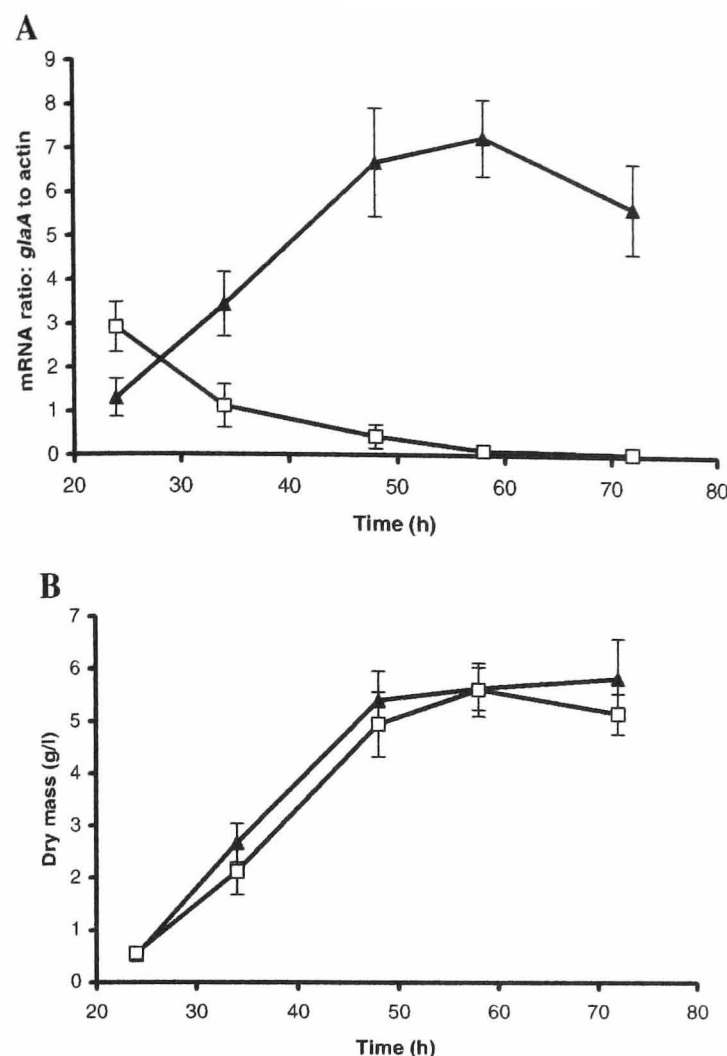


Fig. 2. The effects of antisense *pdiA* under the control of a strong, inducible promoter (*glaA*) on expression of the *glaA* in *A. niger*. A. Two traces are shown: the parental strain AB4.1 (▲) and the inducible antisense *pdiA* strain AS1.1 (□). The time given on the lower axis is in hours after inoculation of the cultures. B. The growth of AB4.1 (▲) and AS1.1 (□). The data represent averages from three replicates for each time-point.

glaA promoter with that of the constitutive *gpdA* (resulting in *A. niger* strain ASG67) to drive expression of the antisense *pdiA* gene. There was a lower level of secreted glucoamylase and *glaA* mRNA in the antisense strain (Fig. 3A and B) but, unlike the situation with the *glaA* promoter, there was still a significant amount of glucoamylase present. This latter difference may reflect the relative strength of the *gpdA* promoter to the *glaA* promoter and their differing roles within the metabolism of the fungus. There was no significant difference in the growth of the parent and the constitutive antisense strain (Fig. 3C).

Evidence for increased processing of the 20 bp 'intron' in the *hacA* mRNA after DTT treatment, relative to control and antisense *pdiA* (*glaA* promoter-driven) strains, is shown in Fig. 4. Also, a size difference between the unprocessed and processed mRNA was detectable, approximately 1 h after DTT treatment, by Northern blotting due to the difference in their 5'- untranslated regions

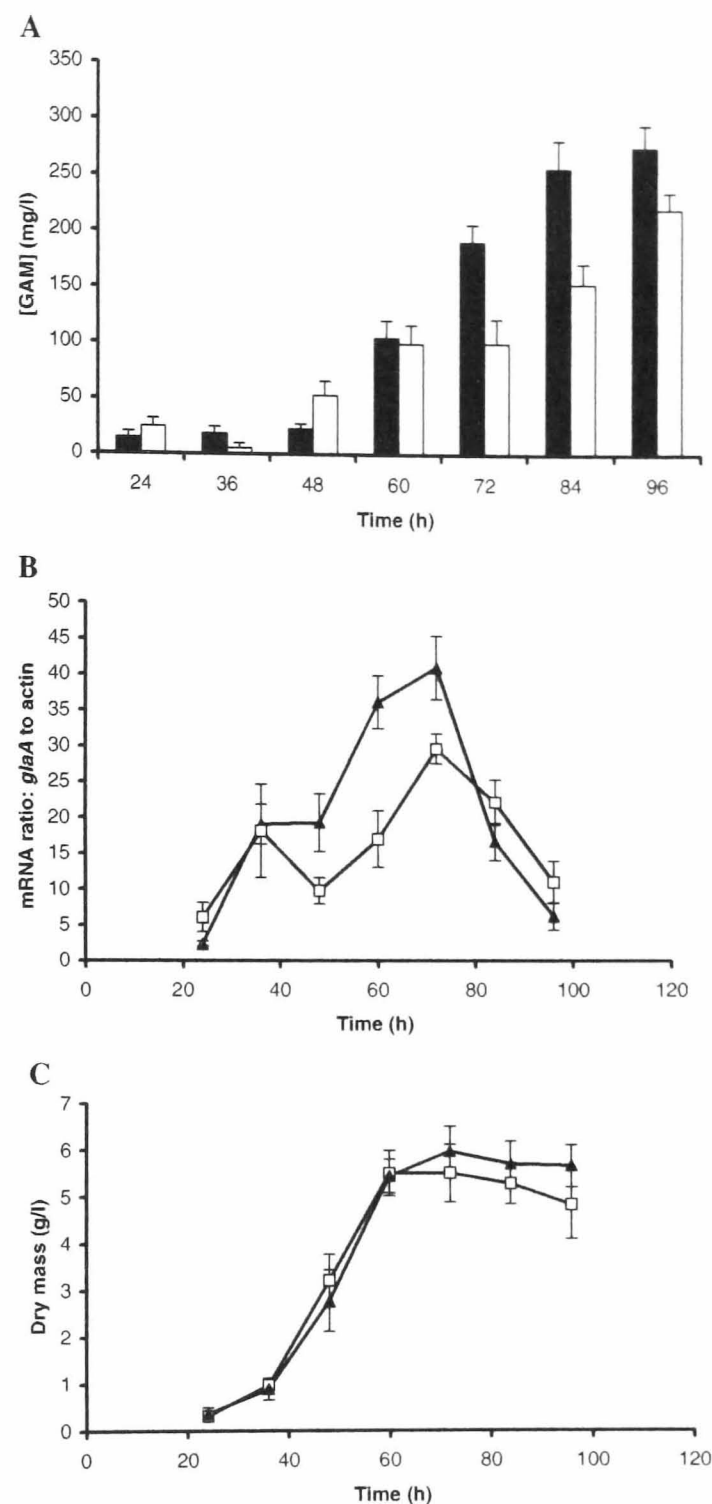


Fig. 3. The effects of antisense *pdiA* under the control of a constitutive promoter (*gpdA*) on expression of *glaA* in *A. niger*. A. The secreted level of glucoamylase enzyme (GAM) from the parental strain AB4.1 is shown as black columns while the same from the constitutively expressed antisense *pdiA* strain, ASG67, is shown in white columns. B. The corresponding mRNA levels of *glaA*:actin in the parental strain, AB4.1 (▲) and the constitutively expressed antisense *pdiA* strain ASG67 (□). The time given on the lower axis is in hours after inoculation of the cultures. The data represent averages from three replicates from each time-point. C. Accumulation of dry mass of AB4.1 (▲) and ASG67 (□).

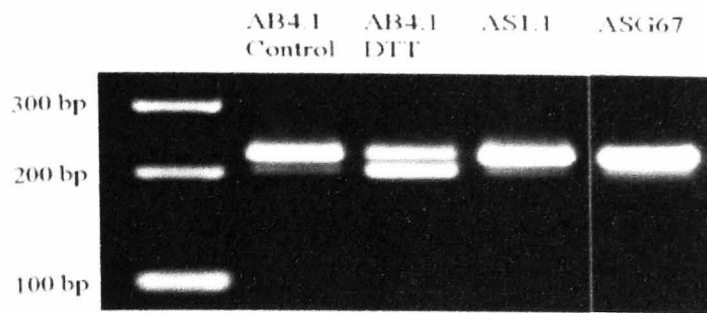


Fig. 4. RT-PCR analysis of the *hacA* transcript across the 20 bp intron of *A. niger* AB4.1 (parental strain) treated with water or DTT, *A. niger* AS1.1 and ASG67 (antisense *pdiA* strains). The *A. niger* AB4.1 control was grown for 44 h and then treated with water and the mycelium harvested after an additional 6 h. The AB4.1 DTT samples were grown as above but treated with 20 mM DTT for 6 h before harvest. The AS1.1 and ASG67 cultures were grown for 48 h and then harvested.

(Mulder *et al.*, 2004). Splicing of the 'intron' within the coding sequence is not detectable by Northern blotting because it only results in a 20 bp difference, whereas Northern blotting can detect the 5'-truncation of 230 bp (Mulder *et al.*, 2004). Processing of the *hacA* mRNA in *A. niger* was detectable by Northern blotting in the DTT-treated cultures but not in water-treated controls or in the antisense *pdiA* strain (data not shown). Reverse transcription polymerase chain reaction (RT-PCR) across the *hacA* mRNA intron (Fig. 4) for untreated parental strain, AB4.1

and for both the constitutive and inducible antisense *pdiA* strains show a very low level of spliced *hacA* mRNA (the faint band below the main band) which is similar in each strain and may represent a background level of *hacA* mRNA splicing. The parental strain treated with DTT shows a much larger component of the spliced *hacA* message. In addition, there was no evidence for transcriptional upregulation of *bipA* in the inducible antisense strain, judged by Northern blotting (data not shown). Together, these results indicate that UPR is not induced in the antisense *pdiA* strains of *A. niger*.

Role of the *glaA* promoter

To assess whether sequences in the promoter of the *glaA* gene play a role in the transcriptional downregulation we analysed two strains of *A. niger* containing the *uidA* reporter gene which encodes β -glucuronidase using the ribonuclease protection assay. The *uidA* gene was placed under the control of two different lengths (1 kb and 2 kb) of the *glaA* promoter and integrated as single-copies at the *pyrG* locus of *A. niger* (Verdoes *et al.*, 1994). Figure 5(B) shows that the 2 kb promoter still exhibits the ability to downregulate control of the *uidA* gene when the strain is exposed to DTT. The truncated promoter is not able to mediate the downregulation effect (Fig. 5B), but

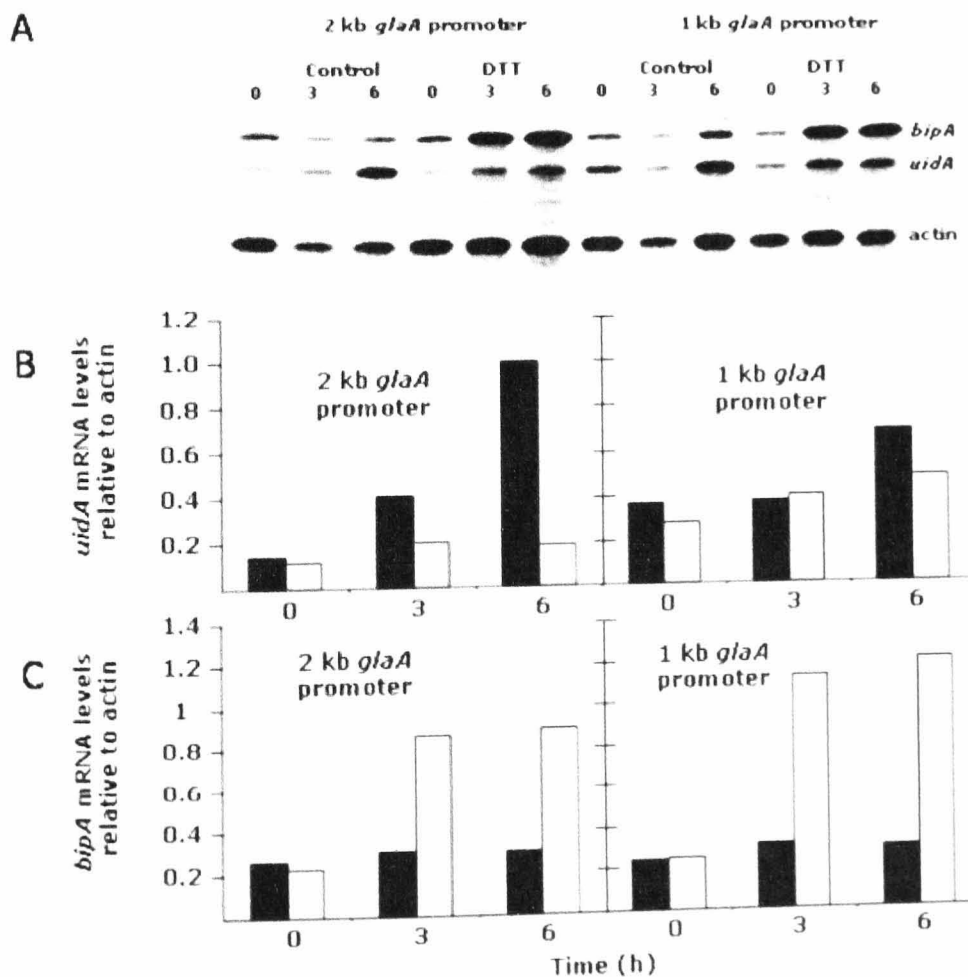


Fig. 5. Ribonuclease protection assay (RPA) of *uidA* reporter constructs driven by 1 kb and 2 kb *glaA* promoters (B), and of *bipA* (C) in the same *uidA* strains. The *A. niger* strains were treated with equal volumes of either water (black bars) or 20 mM DTT (white bars). The mycelium was harvested at 0, 3 and 6 h. The data presented are from a single experiment although it was repeated several times and data collected at various time points. A shows the RPA images that provide the quantified data presented.

the levels of the *uidA* mRNA are lower in this strain, showing that some promoter elements important for transcription are missing. In both instances, the levels of *bipA* mRNA increased in treated samples (Fig. 5C) in a similar way to that in Fig. 1, confirming that the UPR was active.

The data presented to this point show that the steady-state levels of either *glaA* or the reporter *uidA* mRNAs, driven by the *glaA* promoter, were lowered when *A. niger* was stressed by DTT. Although the UPR is induced under these conditions, the use of an antisense *pdiA* strain of *A. niger* showed that the steady-state level of *glaA* mRNA was lowered in the absence of obvious UPR induction. The data point to the effect being brought about at the level of transcription. To eliminate the possibility of mRNA degradation being responsible, we used nuclear run-on to measure the impact of DTT specifically on transcription of *uidA* driven by the *glaA* promoter. The data in Fig. 6(A) show that 20 mM DTT generally suppresses transcription but that the impact is far greater on the *uidA* mRNA than on that of actin. Also, the effect is mediated by a region of the *glaA* promoter between 1 kb and 2 kb upstream of the translational start. UPR was confirmed in this system in response to the DTT treatment (Fig. 6B).

Discussion

We have shown that a mechanism exists in *A. niger* that lowers the level of *glaA* transcripts under conditions of ER stress. The rate of lowering of the *glaA* mRNA levels under DTT stress is close to that of the half-life of degradation,

suggesting that high levels of unfolded proteins in the ER may lead to the transcriptional downregulation of the *glaA* gene. The same was not seen with two genes encoding non-secreted proteins but a lowering of the transcript level of *pepA* encoding the secreted aspergillopepsin was apparent. A system which affects the transcription of genes encoding secreted proteins has been described in the bacterium *Erwinia carotovora* (Vincent-Sealy *et al.*, 1999). The mechanism by which feedback to genes encoding secreted proteins occurs has not been elucidated and, in *Erwinia*, the effects can vary, involving up or downregulation of transcription depending on the particular genes involved. The saprophytic filamentous fungi secrete enzymes to degrade organic polymers and it is possible that the phenomenon has evolved in these organisms as a specific defence against unfolded protein stress because of the high flux of proteins destined for secretion through the ER. The apparent transcriptional downregulation of genes encoding secreted proteins has also been observed in *T. reesei* (Pakula *et al.*, 2003) where it was termed RESS (repression under secretion stress) and in *A. thaliana* (Martinez and Chrispeels, 2003). Martinez and Chrispeels demonstrated in a global analysis of gene expression under separate DTT and tunicamycin stress that most of the genes that were downregulated contained signal sequences targeting the proteins to the cell wall or to extracellular locations. Their study also showed evidence for the induction of the UPR as several chaperones, foldases and other proteins involved in ER function were upregulated. However, both

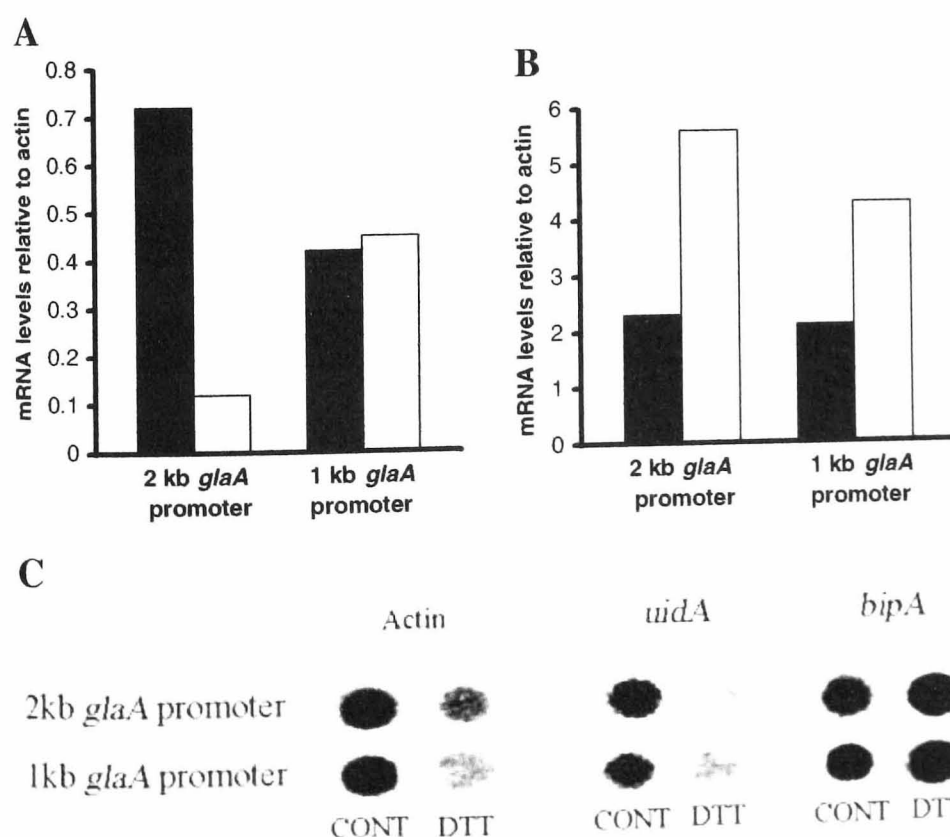


Fig. 6. Nuclear run-on for *uidA* (A) and *bipA* (B) together with some supporting blot data (C) 1 h after treatment with either water (control – black bars) or 20 mM DTT (white bars). A. Showing the effect of water (black bars) or DTT (white bars) on transcription of *uidA* relative to actin. B. Showing the same for *bipA*. *glaA* p refers to the *glaA* promoter of the defined length (1 kb or 2 kb). Data for the Figures are from one experiment although repeats showed the same effects.

of these studies use chemical agents to induce the UPR and have not shown that the transcriptional downregulation of genes encoding extracellular proteins can occur separately to the UPR. Also, ours is the first study to show clearly that the effect is transcriptional, rather than being based on steady-state transcript levels.

The use of DTT to stress the ER is widespread in investigations of the UPR but DTT has a variety of other effects on the cell. Our own research (Watson *et al.*, 2000) and those of Travers *et al.* (2000) and Martinez and Chrispeels (2003) have shown that DTT can affect a wide range of genes, some of which may not be closely related to the phenomenon under study. We have previously shown that antisense *pdiA* strains exhibit lowered levels of foldase activity and lowered levels of secreted glucoamylase (GAM) (Ngiam *et al.*, 2000). We show here that this effect in an antisense *pdiA* strain of *A. niger* is probably caused by a lowering of *glaA* mRNA levels but that this is not mediated by UPR involving splicing of *hacA* message.

The first antisense construct used was driven by the *glaA* promoter which is strongly inducible by starch. As we are also measuring the transcriptional response of the indigenous *glaA* gene to the presence of the antisense *pdiA* mRNA the effect may have been caused by transcription factor titration. However, the presence of between four and six extra copies of the *glaA* promoter (Ngiam *et al.*, 2000) is not expected to affect the transcriptional activity of the *glaA* gene by the titration of essential transcription factors because Verdoes *et al.* (1994) have shown that up to 20 copies of the *glaA* promoter can be incorporated into the genome of *A. niger* before titration effects are manifested. We have used the antisense *pdiA* under the control of the constitutive *gpdA* promoter to ensure the validity of the data obtained with the *glaA*-driven *pdiA* antisense construct. Riboprobe detected the *pdiA* mRNA in the control strain but did not detect either the sense or antisense *pdiA* transcripts in the antisense *pdiA* strains of *A. niger*. Although it has not been possible to delete the *pdiA* gene in *A. niger*, deletion of the other two known *pdiA*-related genes, *tigA* and *prpA*, has been reported (Wang and Ward, 2000). It is not known if the three PDI-family proteins have overlapping functions but *pdiA* may be essential suggesting that the antisense *pdiA* construct is likely to cause stress in *A. niger* that cannot be completely compensated by either TigA or PrpA. It is possible that introduction of the antisense constructs has caused other effects, possibly through ectopic integration of the construct at a locus which is important for the control of transcription in genes which encode secreted proteins. This would then be reflected in a downregulation of transcription from the genes examined (*glaA* and *pepA*) but would not be directly related to the effects of the antisense mRNA. The results obtained from the DTT

experiments, and the fact that we have observed a downregulation in two genes encoding secreted proteins, argues against such possibilities.

We showed that transcriptional downregulation of the *uidA* reporter gene was mediated through a region of the *glaA* promoter between 1 and 2 kb upstream of the translational start point. This, in itself, provides strong evidence that the effect is truly transcriptional rather than a function of mRNA stability. We confirmed this by employing nuclear run-on studies which demonstrated that *de novo* mRNA synthesis driven by the 2 kb *glaA* promoter is significantly reduced under DTT stress. Thus, it appears that DTT stress, under conditions that induce the UPR, leads to the transcriptional downregulation of the *glaA* gene and the effect is mediated by the *glaA* promoter. The steady-state *glaA* mRNA level is also lowered in a strain of *A. niger* where antisense *pdiA* is expressed, and where there is no apparent induction of the UPR. It is therefore possible that the transcriptional downregulation effect is mediated separately from the UPR.

The UPR has been well characterized in both yeast and mammalian systems and is probably the most well understood of all the feedback mechanisms which allow signal transduction from the ER when unfolded proteins are present. The signal transducer in this case is known as IRE1 and the encoding gene was first isolated in yeast (Cox *et al.*, 1993) but homologues have also been isolated from mammalian systems (Wang *et al.*, 1998). IRE1 dimerizes and autophosphorylates when unfolded proteins are present in the ER (Shamu and Walter, 1996) and this, along with the recruitment of a tRNA ligase (Sidrauski *et al.*, 1996) allows the splicing of *hacA* mRNA to occur. The second mechanism that is well understood is translational control in mammalian cells in response to ER stress which involves the protein PERK (Harding *et al.*, 1999). This protein bears similarity to IRE1, especially in its ER lumenal and transmembrane domains. Neither the existence of a functional PERK homologue nor attenuation of translation in response to ER stress has been demonstrated in filamentous fungi. The transcriptional downregulation effect found in *A. niger* may be a different method to solve the same basic problem: how to prevent the entry of nascent protein chains into the ER under conditions when such proteins would be unlikely to fold correctly.

The transcriptional downregulation mechanism that we have detailed is possibly not mediated via *hacA* mRNA processing as there is no evidence for this in the Northern blots for the antisense strains, whereas splicing is evident in cultures treated with DTT. It was, however, possible that the Northern blotting analysis was not sensitive enough to pick up very low levels of processed *hacA* mRNA. The RT-PCR has shown that there are very low levels of spliced *hacA* mRNA present in the wild-type strain and in

the inducible antisense *pdiA* strain of *A. niger* which suggests that the UPR could be active at a very low level in these strains. Because there is no obvious difference in the amounts of spliced *hacA* in the strains (accepting the semi-quantitative nature of the RT-PCR), it may be that the spliced form represents a background level of processing that is always present, possibly to maintain the normal levels of chaperones and foldases in the ER. This does, however, assume that very low levels of the HacA protein would be sufficient to induce transcription of further components of the pathway. It is also possible that there is another transcription factor which acts in a similar way to HacA. It has not until very recently (H. Mulder, unpubl.) been possible to produce strains of *A. niger* which are deleted for *hacA* to facilitate the exploration of such mechanistic possibilities despite several attempts in both *T. reesei* and *A. niger*.

We have shown that a truncated (1 kb) *glaA* promoter-driven construct is not subject to transcriptional down regulation under DTT stress. There was a significant difference in the levels of gene expression between the full-length and truncated promoters, confirming that other essential functions of the promoter have been compromised by the truncation. The requirement now is to pinpoint the area of the promoter which is important in mediating the effect and elucidate the mechanism by which the promoter activity is reduced.

Experimental procedures

Strains and plasmids

The *A. niger* strains used were AB4.1 which is a *pyrG*⁻ strain that is auxotrophic for uridine (van Hartingsveldt *et al.*, 1987) and AS1.1 (Ngiam *et al.*, 2000). The AS1.1 strain of *A. niger* is a multicopy transformant containing an antisense *pdiA* cDNA under the control of the *glaA* promoter and the *Aspergillus nidulans trpC* terminator. In addition, a new strain of *A. niger* was produced called ASG67. *A. niger* AB4.1 was cotransformed with plasmids pGPDAS and pAB4.1 (van Hartingsveldt *et al.*, 1987) which contains a functional copy of the *pyrG* gene from *A. niger*, using the method of Punt and van den Hondel (1987). The plasmid pGPDAS was constructed by PCR amplification of a section of the *pdiA* gene (Ngiam *et al.*, 1997) corresponding to coordinates +118 to +1797 with primers containing *Bsp*HI restriction sites. This fragment was digested with *Bsp*HI and then inserted into pAN52-1 (Punt *et al.*, 1987), containing the *gpdA* promoter and the *A. nidulans trpC* terminator, which had been digested

with *Nco*I. The plasmid is shown in Fig. 7. The transformants were screened by PCR for the presence of the pGPDAS and any positives taken to single spore using a Singer micromanipulator. Primer sequences and PCR amplification conditions are shown in Table 1. The two strains containing the *uidA* gene under the control of either 1 or 2 kb *glaA* promoters, GUS64ÄS and GUS64ÄB were constructed as detailed in Verdoes *et al.* (1994).

Growth and maintenance of cultures

The *A. niger* strains were maintained on potato dextrose agar slopes (Oxoid) with a supplement of 10 mM uridine (Sigma) for AB4.1. Slopes were incubated at 30°C until the cultures had conidiated and were made fresh for each experiment. Spores resuspended in 0.1% (v/v) Tween 20 (Sigma) were used to inoculate slopes and liquid cultures, with an inoculum density of 1×10^5 spores per millilitre being used for liquid cultures. Starch (S)-containing ACMS/N/P medium (Archer *et al.*, 1990) was used for all experiments involving liquid culture except for medium exchange studies which are described below. *A. niger* AB4.1 cultures were supplemented with 10 mM uridine. Cultures were grown in 100 ml of medium in 250 ml conical flasks at 25°C and shaken at 150 r.p.m. In the DTT stress experiments, AB4.1 cultures were grown for 44 h before addition of 1 ml of 2 M DTT solution to give a final concentration of 20 mM. Control cultures had an equivalent volume of sterile water added.

Harvest of material

Mycelia were harvested through two layers of Mira cloth (CalBiochem) and flash frozen under liquid nitrogen. The mycelia were then ground under liquid nitrogen to a fine powder before being freeze-dried in an Edwards Modulyo freeze drier (Edwards).

Nucleic acid manipulations

Total RNA was extracted from 100 mg freeze-dried, ground mycelia using the RNEasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was quantified by reading absorbances at 230, 260 and 280 nm on a UVikon 850 spectrophotometer (Kontron Instruments). Ratios of over 2.0 for the 260 nm:280 nm readings were accepted as being indicative of good quality RNA. Quality was also assessed by running samples on 7% (w/v) formaldehyde gels (Sambrook *et al.*, 1989). For Northern blotting, 5 µg RNA per lane was run on a 7% (w/v) formaldehyde gel in MOPS running buffer (Sambrook *et al.*, 1989) for 16 h at 25 V. Samples were prepared using Sigma RNA loading dye (Cat.# R4268). After

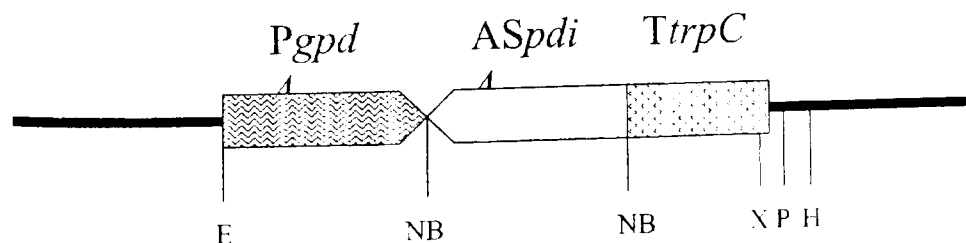


Fig. 7. Layout of pGPDAS (constitutive anti-sense *pdiA* construct). The amplified *pdiA* cDNA was inserted into *Nco*I cut pAN52-1 as a *Bsp*HI cut fragment. E, *Eco*RI; N-B, *Nco*I-*Bsp*HI half sites; X, *Xba*I; P, *Pst*I; H, *Hind*III; Pgp d, *gpdA* promoter; Ttrp C, *A. nidulans trpC* terminator; AS, Antisense. Total size of the plasmid is 6.7 kb.

Table 1. Primers used to amplify probes for PCR and conditions used in the amplifications. All amplifications were carried out for 30 cycles with an initial denaturation step of 5 min at 94°C and a final extension of 10 min at 72°C

Gene	Primers	PCR conditions
<i>act1</i>	5'-TCATGATCGGTATGGGTCAG-3' 5'-ACGATGTTGCCGTACAGATC-3'	94°C/30 s 58°C/60 s
<i>bipA</i>	5'-CAGAGACAGGCCACCAAGGACGCT-3' 5'-CGAACTTGGCGCGGGTGAGGGTCT-3'	72°C/90 s 94°C/30 s 55°C/60 s
<i>glaA</i>	5'-ATCTCTGGGAAGAAGTCAAT-3' 5'-CACAATAGAGACGAAGCCAT-3'	72°C/90 s 94°C/30 s 58°C/60 s
<i>hacA</i>	5'-TTGCCATTGTTGACAGT-3' 5'-CATAAGGAGAGACGTCA-3'	72°C/90 s 94°C/30 s 48°C/60 s
<i>pdiA</i> ^{†1}	5'-TGATACCGAGTCTGATGTTA-3' 5'-CTCGATTCTGTTTGCCTAGC-3'	72°C/90 s 94°C/30 s 45°C/60 s
<i>pdiA</i> ^{†2}	5'-GAGCTTCATGAACGAGCACGCTCTCGTGCTT-3' 5'-CAGTTTCATGAGTTCTGCCTTCTCGTCATCTG-3'	72°C/90 s 94°C/30 s 55°C/60 s
<i>pepA</i>	5'-ACACCGGTACCACCCTCATC-3' 5'-CATCAAATCAACATCATCAC-3'	72°C/90 s 94°C/30 s 48°C/60 s
<i>uidA</i> ^{†a}	5'-AATATTTGCGGCCCACTGG-3' 5'-TCTAGATCATTGTTTGCCTC-3'	72°C/90 s 94°C/30 s 51°C/60 s
<i>uidA</i> ^{†b}	5'-CCTCTAGAAAGCGTATGTTACGTCCTGTAGAAACCCCA-3' 5'-CACCACCTGCCAGTCAACA-3'	72°C/60 s 94°C/30 s 55°C/60 s
<i>pglaAN</i>	5'-TACAGTCGGCTACCAATGCTCTC-3' 5'-CGCATTGAGGAAGTCGGTGT-3'	72°C/90 s 94°C/30 s 56°C/60 s 72°C/90 s

pdiA^{†1}: PCR product used as a probe for blots.

pdiA^{†2}: PCR product used for cloning antisense *pdiA* into pGPDAS.

uidA^{†a}: PCR product used as a probe for Ribonuclease Protection Assay.

uidA^{†b}: PCR product used in the DNA dot blot for Nuclear run-on Assay.

pglaAN: PCR product used as a negative control in the Nuclear run-on Assay.

electrophoresis, the gel was washed in five changes of diethyl pyrocarbonate (DEPC)-treated water (Sambrook *et al.*, 1989) for 20 min per wash and then soaked in 50 mM NaOH for 10 min. Transfer to Hybond XL nylon membrane (Amersham-Pharmacia Biotech) was achieved using an Appligene vacuum blotter according to the manufacturer's instructions with 10× SSC (Sambrook *et al.*, 1989) as transfer buffer. After transfer, the blot was soaked in 50 mM NaOH for 5 min and then rinsed in 2× SSC before being allowed to dry in air.

Probes for Northern blots were labelled using the Megaprime labelling kit (Amersham-Pharmacia Biotech) according to the manufacturer's instructions with [α -³²P]-dCTP (ICN Pharmaceuticals). The *glaA* probe was a 637 bp fragment corresponding to coordinates +1059 to +1696 in the sequence of the *A. niger* glucoamylase gene (Boel *et al.*, 1984). The actin probe was a 765 bp fragment corresponding to coordinates +889 to +1654 in the γ -actin gene of *A. nidulans* (Fidel *et al.*, 1988). The *pdiA* probe was a 303 bp fragment corresponding to coordinates +63 to +365 in the sequence of the *A. niger* *pdiA* gene (Ngiam *et al.*, 1997). The *pepA* probe was a 445 bp fragment corresponding to coordinates +1186 to +1631 in the aspergillopepsin gene of *Aspergillus awamori* (Berka *et al.*, 1990). The *bipA* probe was a 445 bp fragment corresponding to coordinates +712 to +1156 of the *A. niger* *bipA* gene (van Gemeren *et al.*, 1997).

Primer sequences are shown in Table 1. All of the probes were amplified by PCR from *A. niger* genomic DNA and purified from agarose-TAE gels using the Qiaquick gel extraction kit (Qiagen) according to the manufacturer's instructions. The initial PCR fragments were checked by sequence analysis to confirm the probes were identical to the original gene sequence.

Blots were prehybridized in Hyb9 hybridization solution (Puregene) for 30 min before the addition of the labelled probe. The hybridization was carried out overnight at 65°C. Blots were washed twice in 2× SSC, 0.1% (w/v) SDS for 15 min at 65°C and then once in 0.1× SSC, 0.1% (w/v) SDS at 65°C for 30 min. Blots were visualized and the band intensities quantified using a Fuji film BAS2000 phosphorimaging system. RNA loadings were normalized using the γ -actin probe.

Reporter strains and growth conditions

Aspergillus niger reporter strains used in this study were transformed with a reporter construct containing the GUS-encoding gene (*uidA*) of *Escherichia coli* under the control of approximately 2 kb or, after digesting with *Sa*I, 1 kb *glaA* promoter of *A. niger*. These reporter constructs, which have been described previously as pGUS64 and pGUS64 Δ S.

respectively, were integrated as single-copies at the *pyrG* locus of *A. niger* (Verdoes *et al.*, 1994). For nuclear run-on transcription assay and ribonuclease protection assay, cultures were grown in 100 ml xylose (X)-based ACMX/N/P medium (Archer *et al.*, 1990), which represses the *glaA* promoter in 250 ml conical flasks at 25°C and shaken at 150 r.p.m. for 44 h. Cultures were then filtered through Mira cloth (CalBiochem) and washed in the same volume of ACM medium with no carbon source. Mycelia were then transferred to ACMS/N/P medium (where the starch induces the *glaA* promoter) that has the same pH as those cultures grown in ACMX/N/P. Cultures were incubated in the ACMS/N/P medium for 6 h before adding the stress agent (DTT) to final concentration of 20 mM. Mycelia were harvested as mentioned previously.

Isolation of nuclei

Nuclei were isolated using the method described by Schuren *et al.* (1993) with minor modifications. Mycelia from control and (20 mM) DTT-treated cultures were harvested and ground in liquid nitrogen. All further steps were performed on ice. The ground mycelia were resuspended (9 g in 27 ml) in isolation buffer containing 10 mM PIPES (pH 6.9), 0.5 M sucrose, 5 mM CaCl₂, 5 mM MgSO₄, 1 mM-PMSF, 0.1% 2-mercaptoethanol. The suspension was filtered through Mira cloth. The filtered suspension was centrifuged for 10 min at 110 g. The supernatant was centrifuged again to pellet the nuclei for 20 min at 4000 g. The pellet was resuspended in 1.5 ml isolation buffer and loaded to 6.5 ml of the same buffer but containing 2.1 M sucrose and centrifuged for 60 min at 161 000 g. The pellet was resuspended in 0.25 ml of buffer containing 50 mM Tris-HCl (pH 8.3), 40% glycerol, 5 mM MgCl₂, 0.1 mM EDTA and immediately stored at -80°C. The isolated nuclei were checked under the fluorescence microscope after staining with ethidium bromide.

Nuclear run-on transcription assay

Nuclear suspensions (100 µl) were thawed on ice and mixed with 100 µl reaction buffer containing 10 mM Tris-HCl (pH 8.0), 300 mM KCl, 5 mM MgCl₂, 5 mM DTT, 1 mM each of ATP, GTP, CTP and 60 µCi [α -³²P]-UTP. The reactions were allowed to run for 30 min at 30°C. The nuclear DNA was degraded by adding 5 U RNase-free DNase (Ambion) for 5 min at room temperature. SDS and NaCl were added to final concentrations of 1% (w/v) and 0.4 M, respectively. The mixture was extracted once with phenol/chloroform and once with chloroform. The RNA was precipitated with one volume isopropanol and centrifuged for 10 min at 10 500 g. The RNA pellet was dissolved in 50 µl Rnase-free water and cleaned through Spin columns-30 (Sigma) following the manufacturer's instructions. Labelled RNA was denatured by boiling for 2 min and immediately used for hybridization.

All DNA fragments were prepared by PCR using genomic DNA of *A. niger* transformant GUS64 and were purified as detailed above. 1.5 µg of DNA fragments for *bipA*, *uidA* and actin were each spotted on to Hybond N⁺ (Amersham-Pharmacia Biotech). The *uidA* fragment corresponds to *uidA* +1 to +621 of the *E. coli uidA* gene (Schlaman *et al.*, 1994). The

bipA and actin gene fragments were as described above. The negative control used was a DNA fragment of 609 bp corresponding to -1402 to -2011 upstream of the *uidA* translation codon in the 2 kb *glaA* promoter fragment. The blots were prehybridized for 15 min at 65°C in HYB-9 hybridization solution. The denatured labelled RNA was added to the prehybridized blot and hybridization was continued overnight. Blots were washed twice in 2× SSC, 0.1% (w/v) SDS for 10 min. Blots were visualized and the band intensities quantified as detailed above. Faint bands were observed in the negative control, but the background for each band was corrected by subtracting the signal generated from the negative control. Similar observation of low background generated from negative controls when carrying out nuclear run-on assays has been observed in other studies (Gouka *et al.*, 1997).

Glucoamylase assays

The glucoamylase assays were carried out as described previously (MacKenzie *et al.*, 1994) using a glucose oxidase-based assay. The assays were carried out on medium samples taken immediately before harvest of the mycelium and subsequently stored at -20°C. The data are expressed as milligram of GAM present per litre of medium.

Riboprobeing

Single stranded RNA probes were synthesized for the *pdiA* gene using the Universal Riboprobe kit (Promega) to transcribe the *pdiA* PCR product which had been cloned into the pGEMT-easy vector (Promega). Northern blots were produced and hybridized as detailed above but the hybridization temperature was increased to 72°C. Washings were identical in composition and duration to those mentioned above but were also carried out at 72°C.

Rt-PCR

Total RNA was extracted as detailed above and mRNA was isolated from this using the Oligotex mRNA Mini Kit (Qiagen) according to the manufacturer's instructions. First strand cDNA was synthesized using the Omniscript Reverse Transcriptase Kit (Qiagen) with random primers from the Universal Riboclone cDNA Synthesis Kit (Promega) using 1 µg mRNA as template. PCR was carried out using the following primers for amplification of a fragment across the *hacA* intron: 5'-CTTCTCCTACCCTAACTCCT-3' and 5'-TCAAAGAGAGAGAGGGCAC-3'. The PCR conditions were as follows: an initial denaturation of 94°C for 5 min was followed by 30 cycles of 94°C for 30 s, 53°C for 1 min and 72°C for 1 min. The PCR products were visualized by running out on 3% agarose gels with 100 ng ml⁻¹ ethidium bromide.

Ribonuclease protection assay

The RPA was carried out using the RPAIII kit (Ambion) using the PCR amplified probes mentioned in the nucleic acid manipulations section cloned into the pGEM-T Easy vector (Promega) except for the *uidA* probe which was a 552 bp

fragment corresponding to coordinates +1261 to +1812 bp. The final samples were run out on 7% (w/v) acrylamide TBE gels at 200 V for 2 h. The gels were then dried and exposed to a phosphorimaging plate as detailed above.

Acknowledgements

This work was supported by the European Commission Framework 4 Cell Factory – RTD programme (BIO4CT96-0535) and Framework 5 programme (QTRK3-1999-00729) and by the Biotechnology and Biological Sciences Research Council. We gratefully acknowledge a grant to Hashem Al-Sheikh from King Faisal University, Saudi Arabia.

References

- Archer, D.B., and Peberdy, J.F. (1997) The molecular biology of secreted enzyme production by fungi. *Crit Rev Biotechnol* **17**: 273–306.
- Archer, D.B., Jeenes, D.J., MacKenzie, D.A., Brightwell, G., Lambert, N., Lowe, G., *et al.* (1990) Hen egg white lysozyme expressed in, and secreted from, *Aspergillus niger* is correctly processed and folded. *Bio/Technology* **8**: 741–745.
- Berka, R.M., Ward, M., Wilson, M.J., Hayenga, K.J., Kodama, K.H., Carlomagno, L.P., and Thompson, S.A. (1990) Molecular cloning and deletion of the gene encoding aspergillopepsin A from *Aspergillus awamori*. *Gene* **86**: 153–162.
- Boel, E., Hansen, M.T., Hjort, I., Hoegh, I., and Fiil, N.P. (1984) Two different types of intervening sequences in the glucoamylase gene from *Aspergillus niger*. *EMBO J* **3**: 1581–1585.
- Bonifacino, J.S., and Weissman, A.M. (1998) Ubiquitin and the control of protein fate in the secretory and endocytic pathways. *Ann Rev Cell Dev Biol* **14**: 19–57.
- Conesa, A., Punt, P.J., van Luijk, N., and van den Hondel, C.A.M.J.J. (2001) The secretion pathway in filamentous fungi: a biotechnological view. *Fung Genet Biol* **33**: 155–171.
- Cox, J.S., Shamu, C.E., and Walter, P. (1993) Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase. *Cell* **73**: 1197–1206.
- Fidel, S., Doonan, J.H., and Morris, N.R. (1988) *Aspergillus nidulans* contains a single actin gene that has unique intron locations and encodes gamma actin. *Gene* **70**: 283–293.
- van Gemeren, I.A., Punt, P.J., Drint-Kuyvenhoven, A., Broekhuijsen, M.P., van't Hoog, A., Beijersbergen, A., *et al.* (1997) The ER chaperone encoding *bipA* of the black *Aspergilli* is induced by heat shock and unfolded proteins. *Gene* **198**: 43–52.
- Gouka, R.J., Punt, P.J., and van den Hondel, C.A.M.J.J. (1997) Glucoamylase gene fusions alleviate limitations for protein production in *Aspergillus awamori* at the transcriptional and (post) translational levels. *Appl Environ Microbiol* **63**: 488–497.
- Harding, H.P., Zhang, Y.H., and Ron, D. (1999) Protein translation and folding are coupled by an endoplasmic reticulum-resident kinase. *Nature* **397**: 271–274.
- van Hartingsveldt, W., Mattern, I.E., van Zeijl, C.M., Pouwels, P.H., and van den Hondel, C.A.M.J.J. (1987) Development of a homologous transformation system for *Aspergillus niger* based on the *pyrG* gene. *Mol Gen Genet* **206**: 71–75.
- Kawahara, T., Yanagi, H., Yura, T., and Mori, K. (1998) Unconventional splicing of *HAC1/ERN4* mRNA required for the unfolded protein response. *J Biol Chem* **273**: 1802–1807.
- MacKenzie, D.A., Gendron, L.C.G., Jeenes, D.J., and Archer, D.B. (1994) Physiological optimisation of secreted protein production by *Aspergillus niger*. *Enzyme Microb Technol* **16**: 276–280.
- Martinez, I.M., and Chrispeels, M.J. (2003) Genomic analysis of the unfolded protein response in Arabidopsis shows its connection to important cellular processes. *Plant Cell* **15**: 561–576.
- Mori, K. (2000) Tripartite management of unfolded proteins in the endoplasmic reticulum. *Cell* **101**: 451–454.
- Mulder, H.J., Saloheimo, M., Penttillä, M., and Madrid, S.M. (2004) The transcription factor HACA mediates the unfolded protein response in *Aspergillus niger* and upregulates its own transcription. *Mol Genet Genomics* **271**: 130–140.
- Ngiam, C., Jeenes, D.J., and Archer, D.B. (1997) Isolation and characterisation of a gene encoding protein disulphide isomerase, *pdiA*, from *Aspergillus niger*. *Curr Genet* **31**: 133–138.
- Ngiam, C., Jeenes, D.J., Punt, P.J., van den Hondel, C.A.M.J.J., and Archer, D.B. (2000) Characterisation of a foldase, PDIA, in the secretory pathway of *Aspergillus niger*. *Appl Environ Microbiol* **66**: 775–782.
- Pakula, T.M., Laxell, M., Huuskonen, A., Uusitalo, J., Saloheimo, M., and Penttillä, M. (2003) The effects of drugs inhibiting protein secretion in the filamentous fungus, *Trichoderma reesei*: evidence for down-regulation of genes that encode secreted proteins in the stressed cells. *J Biol Chem* **278**: 45011–45020.
- Patil, C., and Walter, P. (2001) Intracellular signalling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. *Curr Opin Cell Biol* **13**: 349–356.
- Punt, P.J., and van den Hondel, C.A.M.J.J. (1987) Transformation of filamentous fungi based on hygromycin and phleomycin resistance markers. *Meth Enzymol* **216**: 447–457.
- Punt, P.J., Oliver, R.P., Dingemanse, M.A., Pouwels, P.H., and van den Hondel, C.A.M.J.J. (1987) Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. *Gene* **56**: 117–124.
- Saloheimo, M., Lund, M., and Penttillä, M. (1999) The protein disulphide isomerase gene of the fungus *Trichoderma reesei* is induced by endoplasmic reticulum stress and regulated by the carbon source. *Mol Gen Genet* **262**: 35–45.
- Saloheimo, M., Valkonen, M., and Penttillä, M. (2003) Activation mechanisms of the HAC1-mediated unfolded protein response in filamentous fungi. *Mol Microbiol* **47**: 1149–1161.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. New York: Cold Spring Harbor Laboratory Press.
- Schlaman, H.R., Risseuw, E., Franke-van Dijk, M.E., and

- Hooykaas, P.J. (1994) Nucleotide sequence corrections of the *uidA* open reading frame encoding β -glucuronidase. *Gene* **138**: 259–260.
- Schuren, F.H., van der Lende, T.R., and Wessels, J.G. (1993) Fruiting genes of *Schizophyllum commune* are transcriptionally regulated. *Mycol Res* **97**: 538–542.
- Shamu, C.E., and Walter, P. (1996) Oligomerization and phosphorylation of the Ire1p kinase during intracellular signalling from the endoplasmic reticulum to the nucleus. *EMBO J* **15**: 3028–3039.
- Shamu, C.E., Cox, J.S., and Walter, P. (1994) The unfolded protein response pathway in yeast. *Trends Cell Biol* **4**: 56–60.
- Sidrauski, C., Cox, J.S., and Walter, P. (1996) tRNA ligase is required for regulated mRNA splicing in the unfolded protein response. *Cell* **87**: 405–413.
- Travers, K.J., Patil, C.K., Wodicka, L., Lochart, D.J., Weissman, J.S., and Walter, P. (2000) Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell* **101**: 249–258.
- Verdoes, J.C., Punt, P.J., Stouthamer, A.H., and van den Hondel, C.A.M.J.J. (1994) The effect of multiple copies of the upstream region on expression of the *Aspergillus niger* glucoamylase-encoding gene. *Gene* **145**: 179–187.
- Vincent-Sealy, L.V., Thomas, J.D., Commander, P., and Salmond, G.P.C. (1999) *Erwinia carotovora* DsbA mutants: evidence for a periplasmic-stress signal transduction system affecting transcription of genes encoding secreted proteins. *Microbiology* **145**: 1945–1958.
- Wang, X.-Z., Harding, H.P., Zhang, Y., Jolicoeur, E.M., Kuroda, M., and Ron, D. (1998) Cloning of mammalian Ire1 reveals diversity in the ER stress responses. *EMBO J* **17**: 5708–5717.
- Wang, H., and Ward, M. (2000) Molecular characterization of a PDI-related gene *prpA*. *Aspergillus niger* var. *Awamori* *Curr Genet* **37**: 57–64.
- Watson, A.J., Worley, J., Elliott, R.M., Jeenes, D.J., and Archer, D.B. (2000) Cloning stress-induced genes from *Aspergillus niger* using polymerase chain reaction-augmented subtractive hybridisation. *Anal Biochem* **277**: 162–165.